Functional characterization of the CXC chemokine-degrading cell envelope protease of *Streptococcus pyogenes*

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Summary

Streptococcus pyogenes is a highly specific human pathogen which has been associated with clinical manifestation of invasive diseases. Invasive infections have a characteristic feature of impaired neutrophil infiltration in the local area of bacterial infection. One pathogenic mechanism behind the lack of neutrophil influx is based on the proteolytic activity of a recently discovered *S. pyogenes* serine protease, streptococcal chemokine protease C (ScpC). ScpC cleaves the major neutrophil attracting chemokine IL-8 and thus renders it functionally inactive to cause transendothelial migration of neutrophils.

This study now identifies ScpC as a cell surface protein which plays an essential role in the invasion process of M3 serotype *S. pyogenes* into human umbilical vein endothelial cells (HUVEC). Biologically active full-length ScpC was expressed in and purified from *Escherichia coli*. Recombinant ScpC (rScpC) coated latex beads were efficiently internalized by HUVEC *in vitro* and ultimately fused with lysosomes, as shown by the co-localization of ScpC coated beads with the late endosomal/lysosomal marker protein LAMP-1, indicating that lysosomes are the terminal cellular compartment for rScpC carriers. By utilizing several rScpC subfragments, it could be demonstrated that the N-terminal PR-domain (putative catalytic domain) *per se* can mediate the internalization of latex beads into HUVEC. In contrast to this, a larger ScpC fragment encompassing both the PR-domain and the flanking A-domain was shown here to be required for the IL-8 degrading activity. By raising a highly specific antiserum against ScpC in rabbit, an IgG fraction was obtained that could efficiently block the interaction of ScpC coated beads with endothelial cells, while having no effect on its IL-8 proteolytic activity. This indicates that the invasion-mediating activity of ScpC is independent of its protease activity. Next, the HUVEC invasion kinetics of a M3 serotype *S. pyogenes* strain and its isogenic Δ*scpC* mutant strain were assessed. The WT strain invaded but subsequently escaped from the endothelial cells via exocytosis whereas the Δ*scpC* mutant strain accumulated within the cells, revealing impaired exocytosis. This suggests that ScpC may play an important role in the transcytosis of *S. pyogenes* across the endothelial monolayer.

Finally, by using the herein generated anti-ScpC antiserum and a set of WT *S. pyogenes* strains as well as their respective isogenic Δ*scpC* mutant strains it could be demonstrated that ScpC is the first LPXTG motif-anchored surface protein with the ability to reassociate back to the bacterial cell surface after its secretion into the extracellular environment.

This study identified novel functions of ScpC, dissected the functional domains, and may thus help to understand the molecular mechanism of action of this genetically conserved large surface protein during *S. pyogenes* infections.

Zusammenfassung

Streptococcus pyogenes ist ein hochspezifischer humanpathogener Krankheitserreger der auch als Auslöser schwerer invasiver Erkrankungen vorkommt. Ein charakteristisches Merkmal invasiver Streptokokkeninfektionen ist die mangelhafte Rekrutierung von Neutrophilen in die bakteriell infizierten Zonen. Dem molekularen Mechanismus dafür liegt die vor kurzem identifizierte proteolytische Aktivität der *S. pyogenes* Serinprotease ScpC (*streptococcal chemokine protease C*) zugrunde. ScpC spaltet das Chemokin IL-8, ein wichtiges Neutrophil-rekrutierendes Botenmolekül, welches durch die proteolytische Spaltung inaktiviert wird.

Im Rahmen dieser Arbeit konnte nun gezeigt werden, dass ScpC eine essenzielle Rolle beim Invasionsprozess von Serotyp M3 Streptokokken in humane Endothelzellen (HUVEC) spielt. Rekombinante ScpC (rScpC) konnte in voller Länge und in biologisch aktiver Form aus *Escherichia coli* Extrakten gereinigt werden. *In vitro* Studien mit HUVEC und rScpC ummantelten Latexpartikeln zeigten, dass rScpC die effiziente Aufnahme in Endothelzellen vermittelt. Durch Kolokalisationsstudien mit LAMP-1, einem späten endosomalen/lysosomalen Markerprotein, wurden Lysosomen als terminale zelluläre Kompartimente des rScpC Transports identifiziert. Durch Generierung unterschiedlicher rScpC Subfragmente und deren Einsatz in Internalisierungsexperimenten konnte die invasionsvermittelnde Domäne eingeschränkt und der N-terminalen PR Domäne (katalytische Domäne, per Def.) zugeordnet werden. Im Gegensatz dazu war ein größeres ScpC Fragment, das sowohl die PR Domäne als auch die flankierende A Domäne umfasst, für die IL-8 degradierende Aktivität notwendig. Mit Hilfe eines im Kaninchen erzeugten spezifischen Antiserums gegen rScpC konnte eine IgG Fraktion gewonnen werden, die effizient die Aufnahme von rScpC ummantelten Partikeln, jedoch nicht die IL-8 spezifische proteolytische Aktivität inhibieren konnte. Dies deutet darauf hin, dass die invasionsvermittelnde Aktivität unabhängig von der proteolytischen Aktivität ist. Als nächstes wurden die Invasionskinetiken eines Serotyp M3 *S. pyogenes* Stammes und seiner isogenen Δ*scpC* Deletionsmutante bestimmt. Während der Wildtypstamm effizient von den Endothelzellen aufgenommen wurde und sie im weiteren Verlauf der Infektion mittels Exozytose wieder verließ, akkumulierte der ScpC defiziente Stamm intrazellulär, was auf eine gestörte Exozytose schließen lässt. Damit spielt ScpC nicht nur bei der Aufnahme in Endothelien sondern auch bei der Transzytose über den Endothelmonolayer *in vitro* eine Rolle.

Ein weiteres wichtiges Ergebnis dieser Arbeit resultierte aus dem Einsatz des hier generierten anti-ScpC Antiserums und zwei *S. pyogenes* Isolaten unterschiedlichen Serotyps und ihrer jeweiligen isogenen ScpC defizienten Mutanten. Es konnte demonstriert werden, dass ScpC

das erste LPXTG Motif-verankerte Oberflächenprotein ist, das nach seiner vollständigen Sekretion in die Umgebung an die Bakterienoberfläche rückassoziiert werden kann.
Diese Studie erlaubt Einblicke in neue Funktionen von ScpC und trägt somit zum erweiterten Verständnis der molekularen Mechanismen dieser großen und genetisch hoch konservierten Serinprotease im Infektionsgeschehen bei.

1 Introduction

The genus *Streptococcus* is made up of non-motile, non-spore forming Gram-positive cocci that grow in chains due to the cell division occurring in one plane with each crosswall made perpendicular to the long axis of the developing chain. Streptococci have an anaerobic metabolism and obtain energy by fermentation. Some are facultative anaerobes and others are strictly anaerobes. The principle end product of fermentation is lactic acid, thus, streptococci are more acid tolerant than most other bacteria. Streptococci are classified on the basis of their hemolysis pattern on the blood agar plate. The Group A streptococcus (GAS, *Streptococcus pyogenes*) belongs to β-hemolytic streptococci, displaying complete lysis of erythrocytes when grown on blood agar. The Lancefield classification system groups the β-hemolytic streptococci according to the carbohydrate antigens present on the surface of the bacterium. *Streptococcus pyogenes* (*S. pyogenes*) is defined by the presence of a polysaccharide composed of a polymer of N-acetyl D-glucosamine and L-rhamnose associated with the cell wall of the bacterium. *S. pyogenes* are further serologically differentiated into M serotypes on the basis of M protein present on the surface of the bacteria. More than 100 distinct serotypes of *S. pyogenes* have been identified based on the N-terminal hypervariable region of M protein (Bisno et al., 2003).

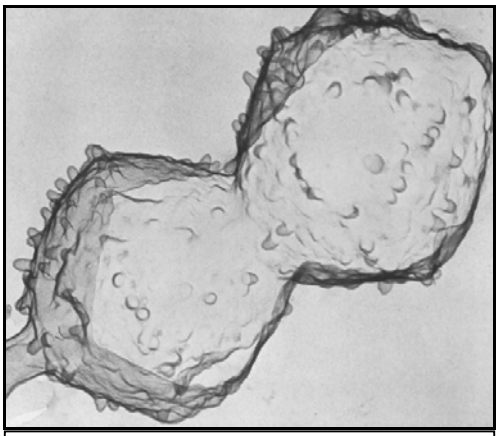


Figure 1. Surface replica of critical point dried whole group A streptococcus representing M-protein projections on the cell surface (Fischetti, 1991).

S. pyogenes is present as a part of the normal flora of the mouth and upper respiratory tract. *S. pyogenes* is a highly specific human pathogen which can cause diseases ranging from mild infections like pharyngitis, erysipelas, scarlet fever and impetigo (pyoderma) to life threatening invasive infections such as streptococcal toxic shock syndrome, myositis and necrotizing fasciitis. *S. pyogenes* infection can also lead to the development of non-suppurative post-streptococcal sequelae including glomerulonephritis and acute rheumatic fever associated with subsequent onset of rheumatic heart disease. Acute rheumatic fever and rheumatic heart disease are the most serious autoimmune sequelae, which are highly predominant among children.

1.1 Epidemiology of *S. pyogenes* infections

The diseases caused by *S. pyogenes* are highly prevalent, with an estimated 18·1 million cases of severe *S. pyogenes* infections, responsible for over 500,000 deaths per year. Among these incidences, the overall mortality due to invasive diseases remains significantly high (Carapetis et al., 2005). An epidemiology study based on the incidence of invasive *S. pyogenes* infections in Canada, conducted from 1995-2001, shows an average of 2.4 cases per 100,000 persons per year, with a 14% death rate (Hollm-Delgado et al., 2005). A similar rate of incidence of invasive *S. pyogenes* diseases was identified from other countries indicating the significant contribution of *S. pyogenes* to serious diseases (O'Brien et al., 2002; Zurawski et al., 1998). Incidence of *S. pyogenes* bacteremia in children under the age of 15 years from Kenya was found to be quite high with 13.0 cases per 10,000 persons per year (Berkley et al., 2005). Various clinical reports have shown that certain M serotypes are associated with specific disease manifestations. *S. pyogenes* strains that cause pyoderma differ in serotype and other biological characteristics from strains classically associated with acute streptococcal pharyngotonsillitis (Wannamaker, 1970). *S. pyogenes* strains of serotype M1 and M3 are more frequently associated with severe invasive *S. pyogenes* infections than other serotypes (Hoge et al., 1993; Hollm-Delgado et al., 2005). Schwartz et al. (1990) have shown that the proportion of invasive *S. pyogenes* infections caused by M1 and M3 serotypes increased over period of 1972-1988 in the USA and these serotypes were more likely to cause fatal infections while M4 and M12 serotypes were found to be less invasive. However, *S. pyogenes* strains associated with invasive diseases can also be isolated from asymptomatic carriers indicating that environmental factors or host factors might contribute to the outcome of invasive diseases (Hollm-Delgado et al., 2005).

1.2 Necrotizing Fasciitis

Necrotizing fasciitis highly contributes to the mortality and morbidity due to *S. pyogenes* infections. The overall mortality of patients with necrotizing fasciitis is 20% - 60%, (Davies et al., 1996; Kaul et al., 1997; Stevens et al., 1989). Necrotizing fasciitis is a rare infection of the deeper layers of skin and subcutaneous tissues, frequently associated with systemic infection symptoms. During deep tissue infection, *S. pyogenes* rapidly progress across the fascial plane involving fascial necrosis, thrombosis in the subcutaneous blood vessels and vascular degeneration. Treatment of necrotizing fasciitis requires the surgical debridement of necrotic

tissue in addition to the use of antibiotics. Several studies have suggested that the lack of an acute inflammatory response may lead to the outcome of deep seated skin infection (Bakleh et al., 2005; Bisno and Stevens, 1996; Cockerill et al., 1998a; Taylor et al., 1999). Histopathological analysis of infected tissue from the patients with necrotizing fascitiis has revealed a correlation between the paucity of neutrophils and the outcome of invasive skin infections (Bakleh et al., 2005). The mortality rate was found to be significantly higher in patients with little or no neutrophil response along with the presence of large concentrations of streptococci in infected tissue. In a primate model of necrotizing fasciitis, out of 13 baboon inoculated intramuscularly with *S. pyogenes*, 2 animals died due to severe invasive *S. pyogenes* infection. The necrotic muscle tissue from animals with severe invasive infection exhibited extensive bacteria along with intravascular aggregation of neutrophils (Figure 2B). However, the neutrophils failed to transmigrate through the endothelial cells and the basement membrane into the infected tissue (Taylor et al., 1999).

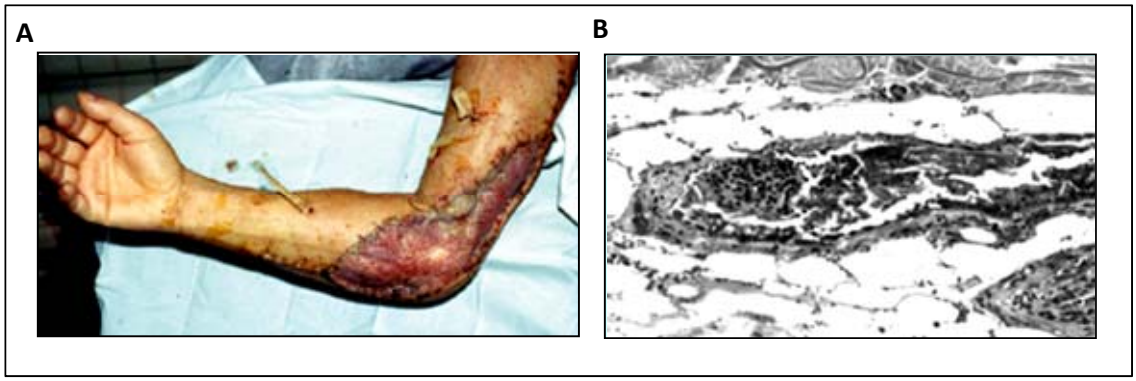


Figure 2. A) Arm of a patient with necrotizing fasciitis showing muscle tissue with extensive necrotic fascia. B) Aggregation of neutrophils in the blood vessel present in the margin of lesion with extensive soft tissue streptococcal infection in a baboon model of necrotizing fasciitis. Images are taken from Hidalgo-Grass et al. (2004) and Taylor et al. (1999), respectively.

The underlying pathogenic mechanism for the lack of inflammatory response is not clearly understood. The outcome of severe invasive diseases due to *S. pyogenes* infection may involve both *S. pyogenes* virulence factors and host susceptibility to infection. *S. pyogenes* expresses several cell surface-associated and secreted factors including hyaluronic acid capsule, M protein, streptococcal chemokine protease C, streptococcal fibronectin binding protein 1, streptococcal pyrogenic exotoxins and streptolysins, to resist clearance by the host innate immune response. These virulence factors perform diverse functions during infection and their expression is regulated to adapt to diverse physiological conditions in the host. The

S. pyogenes virulence determinants involved in impairing neutrophil recruitment can be potential targets for identifying therapeutic agents and/or vaccines against severe streptococcal soft-tissue infections.

1.3 *S. pyogenes* virulence factors involved in impairing phagocytic defence mechanism of the host

Neutrophils are the most important effector cells of the host innate immune response, which provide the first line of defence against infecting pathogens. The recruitment of neutrophils at the site of infection is essential to efficiently resolve the infection by fighting against the invading pathogen. *S. pyogenes* has evolved various virulence mechanisms to avoid clearance by neutrophil-mediated killing. To evade the host immune system, *S. pyogenes* impair neutrophil recruitment, block opsonization with complement proteins and antibodies, employ molecular mimicry and enhance survival inside the neutrophils (Staali et al., 2006; Voyich et al., 2003). Furthermore, *S. pyogenes* can evade killing by neutrophil extracellular traps and induce premature neutrophil apoptosis (Buchanan et al., 2006; Kobayashi et al., 2003). *S. pyogenes* express several virulence factors to enhance survival in the host, the important virulence factors of *S. pyogenes* involved in evading phagocytosis are discussed below.

1.3.1 Hyaluronic acid capsule

The *S. pyogenes* mucoid colony morphology is due to the presence of the capsular polysaccharide, hyaluronic acid which is a repeating unit of D-glucuronic acid and N-acetylglucosamine. Several studies have evidenced the association of highly encapsulated *S. pyogenes* strains with outbreaks of acute rheumatic fever and other severe infections suggesting a role of the hyaluronic acid capsule in *S. pyogenes* virulence (Marcon et al., 1988). The genetic *has* locus required for capsule synthesis is highly conserved among *S. pyogenes* strains. The high expression of hyaluronic acid capsule contributes to the virulence of *S. pyogenes* by enhancing its resistance to phagocytosis. An acapsular mutant of a mucoidal *S. pyogenes* strain of M18 serotype, derived by transposon insertional mutagenesis, was found to be more sensitive to *in vitro* phagocytic killing by neutrophils and less virulent in a mouse model of streptococcal infection (Wessels et al., 1991). Moses et al. (1997) further confirmed the antiphagocytic function of hyaluronic acid capsule by studying the contribution of M protein and hyaluronic acid capsule separately in conferring resistance to phagocytosis. *In vitro* opsonophagocytic assays indicated that a poorly encapsulated strain was more susceptible to killing despite the presence of M protein, while a highly encapsulated strain

survived in 10% serum. Another study with M18 and M24 serotype of *S. pyogenes* indicates that the presence of capsule on the surface of *S. pyogenes* confers protection to phagocytosis despite being equally opsonized with C3 (Dale et al., 1996). Thus, the mechanism of protection might be due to the function of capsule as a physical barrier that blocks the access of complements proteins deposited on the *S. pyogenes* cell surface to phagocytes.

Hyaluronic acid capsule can play a major role in invasive soft-tissue infections. Schrager et al. (1996) have reported that isogenic acapsular mutant strains of highly encapsulated M18 and M24 *S. pyogenes* entered keratinocytes 1000-5000 fold greater in number than the parental encapsulated strain. The parental encapsulated *S. pyogenes* strains were poorly cell invasive and developed a rapidly spreading soft-tissue infection with extensive local necrosis in a mouse model of skin infection whereas the isogenic acapsular mutant strains developed only minor superficial inflammation. Furthermore, Cywes and Wessels (2001) have demonstrated that the interaction of the hyaluronic acid capsule with CD44 receptor present on keratinocytes triggered cytoskeletal rearrangements that led to the disruption of intercellular junctions, thus enhancing translocation of highly encapsulated *S. pyogenes* across the epithelial cell layer into deeper tissues. This observation further supports the important role of hyaluronic acid capsule in enhancing *S. pyogenes* pathogenicity to cause severe invasive infections.

1.3.2 M protein

M protein is an important cell wall-bound virulence determinant of *S. pyogenes*, which was first identified by Lancefield (1962)**.** The presence of M protein alpha-helical coiled coils extending from the streptococcal surface accounts for its fibrillar morphology under the microscope. M protein has a multi-domain structure with the N-terminal half of the protein highly variable and the C-terminal half conserved among different M serotypes. The streptococcal M protein plays a major role in conferring resistance to phagocytosis by polymorphonuclear leukocytes. M protein may exert its antiphagocytic activity via various mechanisms in the absence of type specific antibodies. It has been demonstrated that M protein interferes with the alternative complement pathway by reducing deposition of C3b protein of the complement system on the *S. pyogenes* cell surface (Bisno, 1979). C3b deposition on *S. pyogenes* is required for complement activation and subsequent phagocytic clearance by neutrophils. *S. pyogenes* with reduced C3b deposition on the cell surface cannot interact with the C3b receptor on phagocytes. Studies of Horstmann et al. (1988) suggested that the binding of M protein with factor H, a regulatory protein of the alternative complement

pathway, can be another route of bacteria-induced impairment of complement activation and phagocytic clearance. Factor H prevents C3 activation rendering C3b susceptible to cleavage by factor 1, thus controlling the deposition of C3b on the surface of bacteria. Also, other studies have reported phagocytosis evasion via M protein-mediated binding to fibrinogen (Fg). Interaction of M protein with fibrinogen restricts deposition of C3, thus preventing complement-mediated opsonization of *S. pyogenes* (Whitnack and Beachey, 1982).

1.3.3 C5a peptidase/ScpA

C5a peptidase, also known as ScpA, is highly conserved among *S. pyogenes* strains. C5a peptidase is a cell surface-anchored serine protease that shares sequence similarity with various cell envelope proteases of Gram-positive pathogens. Analysis of C5a peptidase amino acid sequence revealed the presence of a conserved 31-residue signal peptide, a putative cell wall-spanning region and membrane anchor domain (Chen and Cleary, 1989). C5a peptidase contributes to *S. pyogenes* virulence by retarding complement activation. The complement system helps in fighting infection by either activating the classical or alternative pathway via generating C5 convertase, which proteolytically produces chemotaxin C5a. C5a peptidase cleaves chemotaxin C5a specifically at the His^{67}-Lys^{68} bond near the carboxyl-terminus, thus impairing the binding of C5a to its receptor located on polymorphonuclear leukocytes (PMNs). As a result, C5a peptidase impairs neutrophil influx into the areas of *S. pyogenes* infection allowing bacteria to colonize and subsequently invade into deeper tissues (Cleary et al., 1992). By investigating the persistence of $ScpA^+$ and $ScpA^-$ *S. pyogenes* in a mouse air sac model, Ji et al. (1996) have shown that the function of C5a peptidase is to retard the clearance of bacteria during the early stage of infection. In addition, antibody raised against C5a peptidase can neutralize its proteolytic activity and prevent bacterial colonization suggesting the potential role of C5a peptidase as a vaccine candidate (Ji et al., 1997; O'Connor et al., 1991).

1.3.4 Streptolysins

In addition to other virulence factors, *S. pyogenes* produce two well known cytolytic exotoxins: streptolysin O (SLO) and streptolysin S (SLS). SLO is a well-characterized cholesterol-dependent, oxygen labile bacterial exotoxin. A second exotoxin, SLS is a non-immunogenic, oxygen-stable oligopeptide which is responsible for the characteristic zone of lysis of erythrocytes (beta-hemolysis) surrounding *S. pyogenes* colonies grown on blood-agar medium. SLO can be found in cell free culture medium in the secreted form which can bind to

cell membranes containing cholesterol (Kehoe et al., 1987). It causes membrane damage by forming large transmembrane pores with a diameter of around 30 nm. After initial binding with surface cholesterol, SLO spontaneously oligomerizes to form rod shaped structures which can penetrate the cell membrane to form transmembrane channels (Bhakdi et al., 1985). Purified SLO and SLS are highly potent cytolysins, while sublethal concentrations of these toxins have various subtle effects on target cells**.** *In vitro* experiments using a SLO-deficient mutant strain in the background of both adherent and non adherent *S. pyogenes*, have shown that SLO acts synergistically with *S. pyogenes* surface adhesins to induce membrane injury and enhance the expression of pro-inflammatory cytokines by human karatinocytes (Ruiz et al., 1998). SLO deficient mutants in the adherent background fail to stimulate expression of proinflammatory cytokines by keratinocytes, indicating the key role of SLO-mediated membrane damage in the induction of proinflammatory cytokines.

SLO is required for the translocation of *S. pyogenes* NAD-glycohydrolase (NADase) into human epithelial cells. NAD-glycohydrolase is a bacterial toxin, which augment SLO-mediated cytotoxicity and induce apoptosis of skin keratinocytes. SLO and NAD-glycohydrolase enhance extracellular survival of *S. pyogenes* by blocking the internalization of *S. pyogenes*, as shown by significantly reduced internalization of virulent WT M3 serotype *S. pyogenes* in comparison to isogenic SLO and NAD-glycohydrolase mutant strains (Bricker et al., 2002).

1.3.5 Streptococcal chemokine protease C (ScpC/SpyCEP)

1.3.5.1 Cleavage of chemokine interleukin-8 by ScpC

The migration of neutrophils from the bloodstream into damaged/infected tissue forms the first line of defence against infection. Several studies have suggested that a lack of PMN infiltration might be a major cause of persistence and dissemination of *S. pyogenes* in the host which leads to the outcome of invasive deep seated skin infections (Bakleh et al., 2005; Cockerill et al., 1998a; Taylor et al., 1999). In 2004, Hidalgo-Grass et al. first described a new virulence mechanism, whereby *S. pyogenes* evades phagocytosis by impairing chemokine activity. The *S. pyogenes* cell surface protein, ScpC cleaves the host-derived chemotactic factor interleukin-8 (IL-8), which is the major chemoattractant responsible for the neutrophil activation and migration into the infected tissue. Host cells infected with invasive *S. pyogenes in vitro* stimulated the transcription of IL-8, however very little IL-8 was detected in the culture supernatant of host cells when infected with an invasive *S. pyogenes* strain. *In vitro*

experiments performed with WT M14 serotype *S. pyogenes*, isogenic Δ*scpA* mutant and isogenic Δ*scpC*/Δ*scpA* double mutant demonstrated that WT M14 *S. pyogenes* and the isogenic *ΔscpA* mutant strain can efficiently cleave IL-8 whereas the *scpC* mutant strain did not cleave IL-8 indicating that ScpC is responsible for the IL-8 degrading activity of *S. pyogenes* (Hidalgo-Grass et al., 2006). ScpC specifically cleaves IL-8 between Gln^{59}-Arg^{60} in the C-terminal α helix (Edwards et al., 2005). Cleaved IL-8 lacks 13 amino acids of the C-terminus and is biologically incapable of stimulating neutrophil transmigration. The reduced ability of cleaved IL-8 to stimulate neutrophil chemotaxis was demonstrated *in vitro* with an observation of significantly reduced migration of neutrophils across the transwell (5 μm pore size) in response to cleaved IL-8, as compared to intact IL-8 (Edwards et al., 2005). Middleton et al. (1997) have described that IL-8 lacking its C-terminus is unable to interact with heparan sulfate glycosaminoglycans present on the endothelial cell surface, impairing IL-8 transcytosis and presentation to circulating neutrophils. This in turn leads to reduced neutrophil arrest and transendothelial migration suggesting that cleavage of IL-8 by ScpC may contribute directly to the reduced transendothelial migration of neutrophils. This has been demonstrated recently by Zinkernagel et al. (2008) by showing significantly reduced *in vitro* migration of neutrophils across the human microvascular endothelial cells in response to IL-8 co-incubated with a WT M1T1 *S. pyogenes* strain in comparison to the migration of neutrophils in response to IL-8 co-incubated with isogenic Δ*scpC* mutant strain. This confirms that ScpC can retard the IL-8 dependent transendothelial migration of neutrophils and thus impair neutrophil recruitment into the areas of microbial invasion.

1.3.5.2 Role of ScpC in *S. pyogenes* pathogenicity in a murine model of soft-tissue infection

ScpC enhances *S. pyogenes* virulence in a murine model of streptococcal deep tissue infection by interfering with the host inflammatory response towards infection. This was shown by increased survival of mice after subcutaneous infection with an M14 *S. pyogenes* Δ*scpC*/Δ*scpA* mutant strain as compared to mice infected with either wildtype (WT) M14 serotype *S. pyogenes* or an isogenic Δ*scpA* mutant strain. Mice inoculated subcutaneously with the highly invasive WT M14 serotype *S. pyogenes* developed a deep necrotic lesion and died within 2-4 days. Histopathological analysis of necrotic tissue from mice infected with the WT M14 *S. pyogenes* strain identified very few neutrophils at the site of injection along with intensive streptococcal infection in the fascia. However, analysis of necrotic tissue from mice infected with M14 Δ*scpC*/Δ*scpA* strain identified a large number of infiltrating neutrophils. A

paucity of infiltrating neutrophils in the muscle tissue of mice was observed when inoculated with M14 Δ*scpA* mutant strain, similar to the pathological findings of necrotic tissue of mice infected with the WT parental strain. This suggests that ScpA is unlikely to contribute to a reduced inflammatory response (Hidalgo-Grass et al., 2006). A similar pathological outcome of differential neutrophil infiltration was observed in the skin lesion of mice when injected with a globally disseminated M1T1 serotype *S. pyogenes* and the isogenic *scpC* mutant strain (Zinkernagel et al., 2008).

A previous study has reported ScpC protein as a potential vaccine candidate against streptococcal infections. A comprehensive analysis of surface exposed proteins of highly virulent M23 serotype *S. pyogenes*, identified 17 proteins, of which 14 proteins were recombinantly expressed in *E. coli* and tested for their potential to protect against infection of M23 serotype *S. pyogenes*. By immunizing mice intraperitoneally with recombinant proteins, among the 14 surface exposed proteins of *S. pyogenes*, ScpC encoded by *spy0416* and M protein provided highest protection against streptococcal infection, suggesting the potential role of ScpC as a new protective antigen (Rodriguez-Ortega et al., 2006).

The ScpC-mediated paucity of infiltrating neutrophils is localized to the site of *S. pyogenes* infection. This was shown by differential neutrophil infiltration in lesions generated by injecting the same mouse on opposite flanks with an equivalent inoculum of WT M14 *S. pyogenes* and isogenic Δ*scpC*/Δ*scpA* mutant strain. The myeloperoxidase activity (MPO), measured as a marker for neutrophil activity, was significantly lower in the lesion of mouse injected with WT parental strain in comparison to mice injected with isogenic Δ*scpC*/Δ*scpA* mutant strain. Further, the mice injected with WT *S. pyogenes* developed a larger lesion with necrotic subcutaneous muscle tissue in comparison to lesion generated after inoculating with isogenic Δ*scpC*/Δ*scpA* mutant strain**.** These findings were further supported by the presence of similar level of chemokines in the spleen and serum of mice inoculated with WT M14 *S. pyogenes* and isogenic Δ*scpC*/Δ*scpA* mutant strain despite having differential chemokine degradation rate in the skin of mice (Hidalgo-Grass et al., 2006). The localized recruitment of neutrophils plays a major role in limiting proliferation and systemic spread of bacteria from the initial site of infection, thus, by degrading host chemokines in the infected tissue, ScpC plays a significant role in enhancing *S. pyogenes* pathogenicity.

1.3.5.3 ScpC impairs the function of murine chemokines

In addition to human IL-8, ScpC can also cleave the murine CXC chemokines; KC, macrophage inflammatory protein-2 (MIP-2) and granulocyte chemotactic protein 2 (GCP-2).

KC and MIP-2 are two major neutrophil activators and chemoattractants produced by a wide variety of immune cells in response to infection in mice (Bozic et al., 1995)**.** KC and MIP-2 are murine counterparts of the human chemokine GRO which can act as substituent of human IL-8 in mouse (Hidalgo-Gras et al., 2006). The cleavage site of MIP-2 is analogous to that in IL-8 (Edwards et al., 2005). ScpC cleaves MIP-2 between ^{60}Gln-^{61}Lys in its C-terminus suggesting that the cleavage of chemokines by ScpC is dependent upon the presence of specific amino acids and the overall structural conformation of its ligand. *In vivo* studies have shown that the amount of KC and MIP-2 in the skin biopsies of mice infected with M14 *S. pyogenes* Δ*scpC*/Δ*scpA* mutant was significantly higher than the level measured in the skin of mice infected with WT M14 serotype *S. pyogenes* or the isogenic Δ*scpA* mutant (Hidalgo-Grass et al., 2006). The specific cleavage of murine chemokines by ScpC may lead to the observed lack of inflammatory response in the infected areas in murine model of *S. pyogenes* infection, mimicking the pathological findings of severe deep tissue infections in humans.

1.3.5.4 Reduced neutrophil priming via cleavage of host chemokines by ScpC

Another mechanism by which ScpC retards neutrophil infiltration is via cleavage of chemokines granulocyte chemotactic protein-2 (GCP-2) and growth related oncogene α (GRO α). GROα (CXCL1) and GCP-2 (CXCL6) are important inflammatory mediators, which act as potent neutrophil activating proteins as well as chemoattractants. Both GROα and GCP-2 are CXC chemokines with a conserved ELR sequence motif. GROα (CXCL1) shares 33-40% identity with IL-8. GROα is produced by a variety of cells such as monocytes**,** endothelial cells, fibroblasts, and synovial cells after stimulation with lipopolysaccharide, IL-1, or tumor necrosis factor (TNFα) (Geiser et al., 1993). Although GROα and GCP-2 are powerful activators of neutrophils and inducers of chemotaxis, exocytosis and the respiratory burst in neutrophils, they are somewhat less potent than IL-8. GCP-2 has 5-10 fold lower specific activity than IL-8. Cleavage of GCP-2 and GROα by *S. pyogenes* markedly inhibited neutrophil priming as measured by cell surface expression of integrin CD11b by activated neutrophils. Neutrophils showed higher surface expression of CD11b in response to GCP-2 and GROα pre-incubated with a *scpC* deletion mutant as compared to WT M1T1 *S. pyogenes* strain (Sumby et al., 2008).

1.3.5.5 Biological characteristics of ScpC

A homologous sequence search for *scpC* indicates that it belongs to a family of subtilisin-like serine proteases and shares sequence similarity with other cell envelope proteinases from

lactic acid bacteria. The *scpC* gene encodes a 150 kDa protein which is composed of approx. 1,673 amino acids. *scpC* shares 40% sequence identity with the *scpA* gene of *S. pyogenes* (encoding C5a peptidase that cleaves and inactivates human complement protein C5) and 39% identity with *cspA*. CspA is a homolog of ScpC in group B streptococcus which protects bacteria from opsonophagocytic killing by neutrophils via deposition of fibrinogen cleaved products on the cell surface (Harris et al., 2003). ScpC is a putative multi-domain calcium-dependent serine protease with a C-terminal Leu-Pro-X-Thr-Gly **(**LPXTG) motif to cross link with peptidoglycan layer on the surface of bacteria. ScpC has a predicted N-terminal pre-pro domain (PP) with signal peptide sequence which is required for the translocation of protein across the cell membrane, 3 subtilase domains (PR, A and B/H), cell wall spacer domain (W) and cell wall-anchor domain (AN) containing a membrane spanning region of 17 hydrophobic amino acids, a charged amino acid tail and a highly conserved sorting signal, LPXTG cell wall-anchoring motif (Figure 3). The pre-pro-domain has a predicted cleavage site between Val^{123} and Asn^{124}, for the cleavage during the protein maturation process. Three subtilase like domains are designated as PR-domain, A-domain and B-domain partly fused with the H (helical) domain (Hidalgo-Grass et al., 2006).

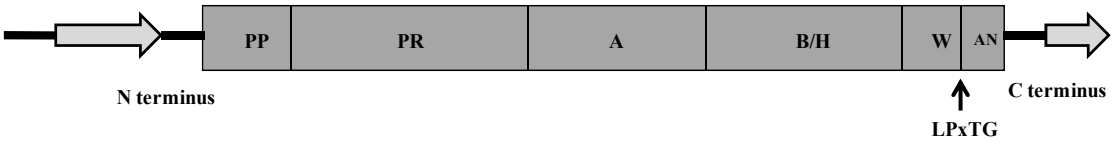


Figure 3. Domain organization of ScpC. Abbreviations indicate: PP, pre-pro-domain containing signal peptide; PR, catalytic domain; A, A-domain which might serve as a domain required for dictating the substrate specificity of ScpC protease; B, stabilizing domain; H, helical domain; W, spacer domain; AN, cell wall-anchoring domain containing LPXTG sequence motif. Arrows indicate genes flanking the ScpC open reading frame (ORF). Left arrow indicates the gene for lactate oxidase and right side arrow indicates ORF of unknown function (Figure adapted from Hidalgo-Grass et al., 2006).

The function of each domain was predicted based on the sequence homology of ScpC with other cell envelope proteases from lactic acid bacteria. The PR-domain is the predicted catalytic domain, containing a conserved catalytic triad of amino acid residues Asp-His-Ser, which exhibit a high degree of similarity with the catalytic subunits of a variety of serine proteases. The A-domain is most likely required for proteolytic activity and may dictate substrate specificity. The putative B-domain along with the H-domain might function as a

spacer and provide overall stability to protease. The W-domain is a cell wall-spanning domain which usually serves as a spacer to keep protease away from the cell surface and the cell wall-anchor domain 'AN' carrying the LPXTG motif (Siezen, 1999). Recent study has suggested that *S. pyogenes* IL-8 degrading activity can be assigned to serine class of proteases as shown by the irreversible inhibition of IL-8 degrading activity of the *S. pyogenes* supernatant with the serine protease inhibitor pefabloc SC. Inhibition of IL-8 proteolytic activity by benzamidine and soybean trypsin inhibitor further suggested that ScpC might share structural and functional similarity with trypsin like serine protease (Hidalgo-Grass et al., 2004).

1.3.5.6 Regulation of *scpC* expression

To adapt to the various locations within its human host, *S. pyogenes* are able to respond to environmental changes by regulating the expression of virulence factors. The expression of virulence factors in *S. pyogenes* is regulated by many stand alone regulators and two component signal transduction regulatory systems. Two-component regulatory systems (TCS) are characterized by having a specific sensor protein located in the cell membrane and a partner response regulator protein. The sensor protein has a kinase activity, which autophosphorylates at a specific histidine residue on the cytoplasmic surface upon receiving a signal from the extracellular environment. This high energy phosphoryl group is then transferred to the partner response regulator, a DNA binding protein, which in turn induces a conformational change in the response regulator. This conformational change alters the binding affinity of the response regulator to a promoter region on the target gene thus regulating the transcription of a specific gene (Chang and Stewart, 1998).

1.3.5.6.1 Regulation of *scpC* expression by *sil*

Although the *scpC* locus is highly conserved among all *S. pyogenes* strains with available genome, ScpC protein expression differs widely among the *S. pyogenes* strains. Edwards et al. (2005) have reported that *S. pyogenes* strains display differential IL-8 degrading activity and the strains possessing IL-8 degrading phenotype gives higher ScpC expression in the *S. pyogenes* supernatant. The *S. pyogenes* strains isolated from blood have higher ability to degrade IL-8 in comparison to non-invasive throat isolates suggesting that *scpC* is differentially regulated and the upregulation of ScpC expression may lead to the invasiveness of the *S. pyogenes* strains**.** Hidalgo-Grass et al. (Hidalgo-Grass et al., 2002) demonstrated the role of the streptococcal invasion locus (*sil*) in regulating the invasive potential of the M14 *S. pyogenes* strain JS95. The *sil* locus contains *silA/B* encoding a TCS and *silD/E* encoding two

ABC transporters. In between *silA/B* and *silD/E*, two highly overlapping open reading frames *silC* and *silCR* are situated. The *sil*CR gene encodes a 17 amino acid residue signalling peptide (CSP) which modulates the virulence of M14 *S. pyogenes* by regulating the expression of ScpC. *silCR* can not be translated in invasive M14 *S. pyogenes* strain JS95 due to the presence of a missense mutation in the start codon. The role of the SilCR signalling peptide in modulating *S. pyogenes* pathogenicity in a murine model of necrotizing fasciitis was observed by co-injecting *S. pyogenes* with SilCR peptide. Inoculation of mice with M14 *S. pyogenes* strain JS95 alone was lethal and engendering necrotic tissue containing a large number of streptococci along with the absence of neutrophils and ultimately death of the animal. However, co-injection of M14 *S. pyogenes* with SilCR triggered the neutrophil influx into the infected area thus restricting bacterial proliferation and systemic bacterial spread (Hidalgo-Grass et al., 2004). SilCR negatively regulates *scpC* transcription by interacting with the two component system, SilA/B. The subcutaneous co-injection of SilCR and M14 *S. pyogenes* in mice downregulates transcription of *scpC* by 10 fold (Hidalgo-Grass et al., 2006). Transcription of *silC* and *silCR,* highly regulated by the SilCR peptide, makes a regulatory circuit that in turn regulates the invasive potential of *S. pyogenes*. SilCR autoregulates the transcription of *silCR* via the two component system *silA/B*, as shown by marked upregulation in the transcription of *silD/E/CR* by the WT JS95 strain when grown in the presence of SilCR. However, in JS95 *silA* mutant strain, SilCR was unable to induce the transcription of *silD/E/CR*. (Eran et al., 2007). The *sil* locus was found in 16% of the invasive *S. pyogenes* isolates of different *emm* types (Bidet et al., 2007) and 18% of *S. pyogenes* strains containing *sil* locus produced SilCR peptide. Although, the *sil* is absent in M1 and M3 serotype *S. pyogenes*, which are predominant serotypes associated with invasive *S. pyogenes* diseases, the impact of SilCR in modulating M14 *S. pyogenes* virulence indicates that it might play an important role in regulating the expression of ScpC in other serotypes.

1.3.5.6.2 Regulation of *S. pyogenes* virulence by two component system *covRS*

A two component regulatory system, *covRS* (also designated as *csrRS*) influences the transcription of 15% *S. pyogenes* genes including several virulence factors. It negatively regulates the expression of important virulence factors i.e. hylauronic acid capsule, SpeB, streptokinase, streptolysin S, and streptodornase. Inactivation of *covRS* results in increased production of these virulence factors thus enhancing *S. pyogenes* virulence in mouse models of invasive disease (Federle et al., 1999; Heath et al., 1999; Levin and Wessels, 1998). Recently, Sumby et al. (2006) suggested the role of *covRS* in a phenotypic switch from non-

invasive to invasive phenotype by regulating the differential transcription of certain virulence factors. By studying the distinct trascriptome of invasive and pharyngeal isolates, it has been shown that a spontaneous generation of a single 7-bp insertion mutation in the gene encoding the sensor kinase component (*covS)* of the two component regulatory system *covRS* leads to the invasive phenotype of *S. pyogenes*. This mutation leads to a characteristic increase in the expression of various virulence factors including hyaluronic acid capsule, SpyCEP, Sic, Mac, streptococcal DNase D2, C5a peptidase and other exoproteins. A marked upregulation (>25 fold) in the transcription of *scpC* (*spyCEP*) by invasive *S. pyogenes* isolates was observed in comparison to pharyngeal isolates, further supporting the contribution of ScpC to the outcome of invasive *S. pyogenes* disease. Additionally, the mutation in *covRS* leads to upregulation of ScpC protein expression as shown by enhanced concentration of secreted ScpC by *covR/S* mutant strain in comparison to WT *S. pyogenes* strain further indicates that *scpC* is negatively regulated by *covRS* (Sumby et al., 2008).

1.4 *S. pyogenes* and the host innate immune response

1.4.1 Neutrophils

The migration of neutrophils from the bloodstream into infected tissue is the key feature of inflammation. Neutrophils, which are normally found in the bloodstream, migrate from circulating blood to the infected tissue in response to an inflammatory signal. The average lifespan of neutrophils is 12 h in blood and 1-2 days in tissue. To reach the infected tissues, neutrophils transmigrate across the endothelium and the basement membrane, a process known as diapedesis. Neutrophil extravasation is a multi-step process which includes tethering, rolling, activation, adhesion and subsequent migration of adherent cells through the intercellular spaces between the endothelial cells to the infected underlying tissue. This process involves a complex system of intracellular cell signaling and regulated interplay between various adhesion molecules on neutrophils and vascular endothelial cells to cause cells trafficking from the bloodstream into the tissue. The tethering and rolling of neutrophils along the endothelium is mediated by the transient interaction between selectins (L, E and P-selectins; cells adhesion molecules) and their carbohydrate ligands (Lawrence et al., 1994; Ley and Tedder, 1995). The rolling of polymorphonuclear leukocytes (PMNs) on the endothelial cell surface facilitates their exposure to chemokines bound on the endothelial cell surface via glycosaminoglycans (Rot et al., 1996). The chemokines trigger the arrest of rolling leukocytes expressing specific receptors by providing a signal through G-protein coupled

receptors that convert the low affinity selectin mediated interaction to high-affinity integrin mediated interaction with intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 present on endothelial cells. To induce the integrin mediated neutrophil arrest, chemokines enhance the integrin avidity to endothelial cell ligands (Chan et al., 2001; Rainger et al., 1997). The leukocyte adhesion to endothelial cells subsequently transmits the signal to leukocytes to undergo cytoskeleton rearrangement that contributes to the outcome of transendothelial migration.

1.4.2 Neutrophil killing mechanism

After reaching the site of infection, neutrophils engulf the microbe by phagocytosis into a vacuole known as a phagosome. Once phagocytosed, the microbe is killed within the phagocyte via both, non-oxidative and oxidative mechanisms (Nathan, 2006). The non-oxidative mechanism involves the discharge of granules containing antibacterial compounds into the phagosome. Neutrophil granules are categorised into primary (azurophilic granules), secondary (specific granules) and tertiary granules (gelatinase granules). The secondary and tertiary granules contain antimicrobial proteins such as lysozyme, lactoferrin, lipocalin, gelatinase and metalloproteases. Metalloproteases facilitate the migration of neutrophils into the tissue by degradation of extracellular matrix proteins (Borregaard and Cowland, 1997). The azurophilic granules contain small antimicrobial peptides, defensins, cathepsin G, proteases, elastase and myeloperoxidase (MPO) (Urban et al., 2006). The oxygen dependent mechanism kills the microbe via respiratory burst, in which reactive oxygen species including superoxide, singlet oxygen, ozone, hydrogen peroxide, hypohalous acids, chloramines and hydroxyl radicals are generated. The respiratory burst has been shown to be essential for the optimal killing of many bacterial species. It involves abrupt non-mitochondrial reduction of oxygen by NADPH-oxidase complex assembled in the phagosomal membrane to generate superoxide (O_2^-) (Babior et al., 1973; Segal and Abo, 1993).

$$\text{NADPH} + 2O_2 \xrightarrow{\text{NADPH Oxidase}} \text{NADP}^+ + 2O_2^- + H^+$$

Superoxide ions are converted to H_2O_2 by superoxide dismutase (SOD) which in turn, is consumed by MPO to form hypochlorous acid (HOCl). It has been shown that HOCl is the strong non-radical bactericidal oxidant produced by the neutrophils (Hampton et al., 1998). Subsequently, the phagocytosis of the microbe induces changes in PMN gene expression

leading to programmed cell death (apoptosis) of PMN which is essential to limit tissue damage and contributes to resolve the bacterial infection. Studies of Kobayashi et al. (2003) have shown that *S. pyogenes* can enhance its survival by altering the PMN apoptosis program as observed the enhanced, premature apoptosis of neutrophils incubated with WT *S. pyogenes* as compared to heat-killed *S. pyogenes* which had very low capacity for inducing PMN apoptosis**.**

1.4.3 Neutrophil Extracellular Traps

In addition to intracellular killing by neutrophils, activated neutrophils can release neutrophil extracellular traps (NETs), which are essential for extracellular killing of bacteria. NETs are composed of antimicrobial proteins and proteases of neutrophil granules along with DNA and histones as a major structural component. NETs are non-membranous traps which kill the microbe by delivering a high local concentration of antimicrobial molecules (Brinkmann et al., 2004). It has been shown that *S. pyogenes* circumvent neutrophil killing by degrading neutrophil extracellular traps (Figure 4). The *S. pyogenes* M1T1 clone is frequently associated with invasive diseases and expresses a phage encoded DNase streptodornase 1 (Sda1). Sda1 enables *S. pyogenes* to survive extracellular killing by degrading the DNA framework of NETs as evidenced by dose-dependent degradation of NETs by WT M1T1 in comparison to M1T1 Δ*Sda1* mutant, where significant quantities of NETs were observed upon co-incubation (Buchanan et al., 2006). Additionally, it has been reported that degradation of IL-8 by ScpC can lead to reduced IL-8 mediated NET production which may aid the bacteria to escape the extracellular killing by neutrophils thus enhancing bacterial survival and spread (Sumby et al., 2008).

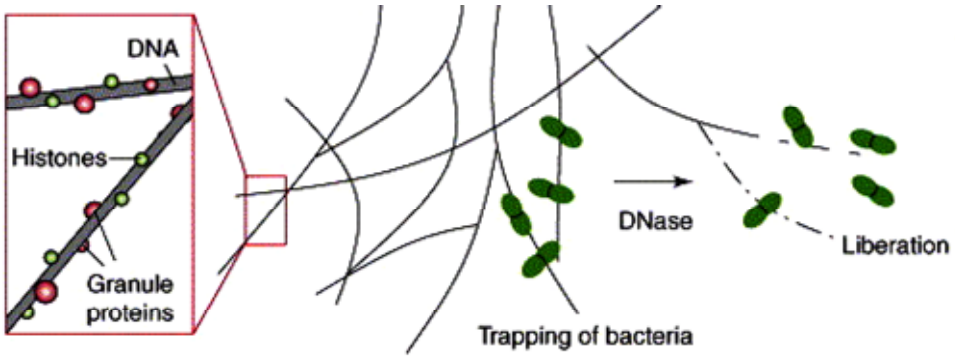


Figure 4. Degradation of NETs by *S. pyogenes*. NETs consisting of DNA backbone, histones and granules. *S. pyogenes* expresses DNases that degrade the DNA-backbone of NETs. Image modified from Wartha et al. (2007).

1.4.4 Interleukin-8

IL-8, also known as neutrophil chemotactic factor-1, is a major host-derived chemokine which acts as an activator and chemoattractant of neutrophils during the host inflammatory response (Bickel, 1993). IL-8 is produced by a variety of cell types during infection, including monocytes, T lymphocytes, neutrophils, fibroblasts, epithelial cells and endothelial cells, upon activation by other pro-inflammatory mediators and cytokines. IL-8 also mediates respiratory burst, granule release, and enhances cellular adhesion of neutrophils to the endothelial cell surface. The activity of IL-8 is mediated through its binding to specific GTP-coupled, seven-helix transmembrane chemokine receptors, CXCRl and CXCR2 on neutrophils. IL-8 is a member of the subclass of CXC chemokines which contains a specific motif of amino acid sequence Glutamic acid-Leucine-Arginine (ELR) immediately before the first conserved cysteine near the N-terminus. Chemokine members of the CXC branch of the family have four invariant cysteines, the first two of which are separated by one non-cysteine amino acid residue designated as X (Strieter et al., 2002). IL-8 is a non-glycosylated protein of 72 amino acids with a molecular mass of 8.4 kDa, which is produced by the processing of a precursor protein of 99 amino acids.

1.4.4.1 Transendothelial migration of neutrophils

During inflammation, circulating leukocytes migrate across the endothelial barrier to reach the site of microbial infection/ tissue injury. IL-8 is a major neutrophil chemoattractant which induces the migration of neutrophils across the inflammed endothelium (Smith et al., 1991). Endothelial cells produce IL-8 constitutively, and show markedly increased IL-8 production

in response to inflammatory signals such as TNF- α and IL-1 (Strieter et al., 1989). To cause the transendothelial migration of leukocytes, tissue derived IL-8 and endothelial cell derived IL-8 is transported to the luminal surface of the endothelial cells (Rot et al., 1996). IL-8 gets immobilized on the luminal side of the endothelial cell surface via highly charged amino acids present at the C-terminus. The IL-8 C-terminal alpha helix mediates transient anchoring of IL-8 on endothelial cells via binding with heparin sulphate glycosaminoglycans present on the endothelial surface. Therefore, an intact C-terminus of IL-8 is necessary for both the transcytosis across the endothelial cells and subsequent binding on the endothelial apical cell surface (Middleton et al., 1997; Webb et al., 1993; Witt and Lander, 1994). Binding of IL-8 on the endothelial cell surface creates a haptotactic chemokine gradient of IL-8 which then triggers the recruitment of leukocyte subsets expressing specific receptors into the area of microbial invasion (Rot, 1992; Rot et al., 1993). The presentation of IL-8 anchored on the apical surface of endothelial cells to circulating neutrophils expressing the chemokine receptors CXC1and CXC2 is responsible for the neutrophil arrest and promigratory activity of IL-8 (Skelton et al., 1999). *In vivo*, due to the continuous flow of blood, soluble chemokines would be washed away and soluble chemokine gradient would rarely persist in the blood stream. Huber et al. (1991) have shown that endothelial cells produce a 77 amino acid variant of IL-8 when stimulated with proinflammatory mediators like cytokines or lipopolysaccharide (LPS). The endogenous IL-8 synthesized by stimulated endothelial cells induces neutrophil attachment and migration across the endothelial monolayer. Various other studies have

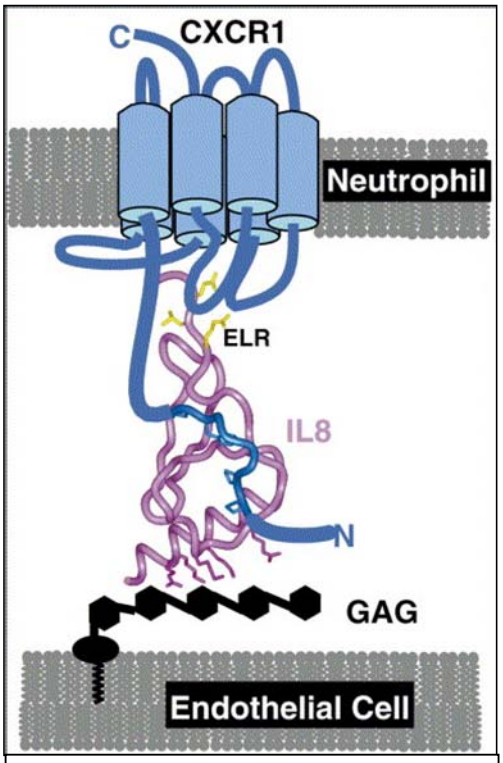


Figure 5. Model showing the binding of IL-8 on the endothelial cell surface via glycosaminoglycans and the presentation of immobilized IL-8 to the CXCR-1 receptor on a passing neutrophil (Skelton et al., 1999).

also shown that chemoattractants bound to the surface of endothelial cells are responsible for inducing the transmigration of neutrophils. In contrast, the soluble IL-8 added externally to the cell culture inhibited adhesion and transmigration of neutrophils (Rot, 1992). Apically bound chemokines on activated human umbilical vein endothelial cells (HUVEC) augmented T cell arrest and subsequent migration through the endothelial cell barrier to the site of inflammation in the absence of soluble chemotactic gradients across the endothelial barrier (Cinamon et al., 2001; Middleton et al., 1997). In contrast, another study has reported that TNF-α stimulated endothelial cells produced two chemokines GRO-α, and MCP-1. GRO-α immobilized on the endothelium mediates monocyte adhesion and arrest to the endothelium under shear flow while a soluble MCP-1 gradient mediates the transendothelial migration of monocytes (Weber et al., 1999).

1.5 Intracellular invasion by *S. pyogenes*

S. pyogenes, once considered as an extracellular pathogen, can efficiently invade and survive within a variety of host cells (Hagman et al., 1999; LaPenta et al., 1994). *S. pyogenes* triggers its own uptake by non-phagocytic host cells during its initial encounter with the host. Although the pathogenic significance of the invasion of *S. pyogenes* into non-phagocytic host cells is not clearly understood, it is possible that an intracellular state may provide a protective niche to bacteria by providing protection from antibiotic therapy and host defence mechanisms that enables *S. pyogenes* to persist in the host and invade deeper tissues. Antibiotic treatment with penicillin and clindamycin can efficiently eradicate extracellular *S. pyogenes*, however it is unable to kill intracellularly residing bacteria, which might be the major cause of antibiotic treatment failure for streptococcal infections. Brandt et al. (2001) have shown that 80% of pharyngitis patients had relapse of infection with the same *S.*

pyogenes strains which were originally isolated, even after vigorous penicillin treatment. The intracellular presence of viable *S. pyogenes* in pharyngeal epithelial cells collected from patients with tonsillitis and the tonsils of asymptomatic *S. pyogenes* carriers suggests that intracellular *S. pyogenes* constitute a reservoir of bacteria capable of causing recurrent *S. pyogenes* infections (Osterlund and Engstrand, 1997; Osterlund et al., 1997). *S. pyogenes* can also reside intracellularly in host phagocytic cells like macrophages, monocytes and neutrophils. Intracellular viable streptococci were isolated from macrophages in skin biopsies of patients with severe invasive *S. pyogenes* soft tissue infection. *In vitro* invasion of *S. pyogenes* into macrophages showed that the intracellular viable *S. pyogenes* were able to replicate extracellularly after the removal of antibiotics. Also, intracellular *S. pyogenes* were frequently isolated from newly involved non-inflamed tissue suggesting that internalization of *S. pyogenes* by host cell may lead to the invasion of the deeper tissues (Thulin et al., 2006).

Invasion of host cells is a multi-factorial process involving the interaction of specific cell surface exposed virulence factors of *S. pyogenes* with host components. *S. pyogenes* enhances intracellular survival via various strategies to evade lysosomal killing, which serves as an important intracellular innate defense function. Lysosomes are the terminal compartments in the cellular endocytic pathway which kill and degrade the phagocytosed microbe upon fusion with the phagosome. Lysosomes have an acidified lumen with pH 4.5, maintained by the proton pump activity of the vacuolar ATPase, and digestive enzymes including proteases, lipases, lysozyme and nucleases that work optimally at acidic pH (Mellman et al., 1986). To enhance intracellular survival, *S. pyogenes* are able to inhibit the fusion of phagosome with lysosome, escape from phagosome into the cytoplasm and enhance survival inside the phago-lysosomes. M1 serotype *S. pyogenes* expressing surface-associated M or M-like proteins can survive and replicate inside the neutrophils via specifically inhibiting the fusion of azurophilic granules with *S. pyogenes* containing phagosomes (Staali et al., 2006; Staali et al., 2003). Also, *S. pyogenes* are able to combat the oxidative stress by upregulating the expression of genes such as such as alkylhydroperoxidase *(ahpC)*, alkylhydroperoxidase reductase *(nox1)* and glutathione peroxidase *(bsaA)* which encode proteins that detoxify reactive oxygen species and other free radicals in neutrophils (Voyich et al., 2003).

1.5.1 Virulence factors involved in the invasion of *S. pyogenes* into host cells

The *S. pyogenes* surface protein Sfb1 (also known as PrtF1) is an essential factor for adherence to and invasion into human epithelial (HEp2) cells. Epidemiology studies have

shown that the *sfb1* gene is present in 50-70% of *S. pyogenes* isolates (Neeman et al., 1998; Talay et al., 1992). Sfb1 binds the extracellular matrix protein fibronectin (Fn) with high affinity. Pre-incubation of Sfb1-carrying streptococci with soluble Fn efficiently promoted bacterial adherence to epithelial cells which is indicative of the role of Sfb1 in the colonization process during infection (Valentin-Weigand et al., 1994). The binding of Sfb1 to Fn is a prerequisite for adhesion to and invasion into non-phagocytic host cells. Fn acts as a bridge between Sfb1 present on the *S. pyogenes* cell surface and the $\alpha_5\beta_l$ integrin receptor on HEp2 cells. Molinari et al. (1997) have demonstrated that the presence of Sfb1 on the *S. pyogenes* surface is sufficient to trigger invasion into HEp2 cells as shown by efficient internalization of Sfb1 coated latex beads by epithelial cells. Sfb1 mediates adherence to epithelial cells via its C-terminal fibronectin-binding repeat region which is a prerequisite for the subsequent invasion process. The lack of the fibronectin-binding domains impairs the adhesion and internalization of recombinant Sfb1 protein by epithelial cells. The SfbI spacer domain also triggers the invasion of streptococci into the eukaryotic cells. Two distinct N-terminal regions of fibronectin co-operatively interact with the two C-terminal fibronectin binding domains of Sfb1, a process that subsequently triggers the invasion of *S. pyogenes* into epithelial cells (Talay et al., 2000).

Rohde et al. (2003) have shown that *S. pyogenes* follows distinct pathways for invasion into host cells depending upon the expression of specific virulence proteins. Sfb1 expressing *S. pyogenes* strains follow a caveolae-mediated endocytic pathway to gain entry into epithelial as well as endothelial cells. Sfb1 expressing *S. pyogenes* strains trigger the formation of large membrane invaginations in the host cells via fibronectin-mediated interaction with the cellular integrin $\alpha_5\beta_1$. Sfb1 expressing *S. pyogenes* induce integrin clustering at the site of entry which results in recruitment of host cell caveolae towards the cytoplasmic membrane. Recruitment of caveolae subsequently leads to streptococcal uptake into distinct compartments called as caveosomes. *S. pyogenes* thereby evades the fusion with lysosomes and bypasses the classical endocytic pathway of eukaryotic cells. Incubation of epithelial cells with purified recombinant Sfb1 protein was sufficient to trigger the uptake process by formation of membrane invaginations in the host cell as shown by clustering and uptake of Sfb1 coated gold particles. In contrast, Sfb1 negative M1 *S. pyogenes* uses a distinct pathway to invade host cells. Binding of Sfb1-negative *S. pyogenes* to host cell microvilli triggers cytoskeletal rearrangements that induce the formation of membrane protrusions by epithelial cells to engulf the microbe (Rohde et al., 2003).

M-protein has also been shown to participate in the interaction with eukaryotic cells. Invasion of *S. pyogenes* into epithelial cells was triggered by binding of M1 protein to the extracellular matrix proteins fibronectin or laminin. Inactivation of the *emm1* gene reduced the binding of *S. pyogenes* by 88% indicating that interaction of M1 protein with Fn is a prerequisite to mediate adherence and internalization of M1 serotype *S. pyogenes* into A549 cells. Also, the invasion of epithelial cells by a highly virulent M1 clone was dependent upon exposure of bacteria to fibronectin or synthetic peptide containing RGD amino acid sequence (Cue and Cleary, 1998). The role of M6 protein in invasion of *S. pyogenes* into human epithelial cells was confirmed by Fluckiger et al. (1998). The M^+ strain invaded a human pharyngeal cell line at a significantly higher rate than isogenic M^- strains of *S. pyogenes*. Another report has shown that both M protein and SfbI contribute significantly to the invasion of epithelial cells and require the activation of the integrin-linked kinase signaling pathway (Wang et al., 2006a). The expression of heterologous Sfb1 in the *S. pyogenes* strain of M1 serotype significantly enhanced the potential of *S. pyogenes* to invade epithelial cells and resistance to phagocytosis suggesting the significant contribution of Sfb1 in enhancing the virulence of *S. pyogenes* (Hyland et al., 2007). LaPenta et al. (1994) have reported that streptococcal pyrogenic exotoxin A (SpeA) might contribute to the invasion process of *S. pyogenes* into host cells as evidenced by the higher frequency of invasion of M1 strain containing *speA* gene into epithelial cells in comparison to a *speA* negative M1 strain.

Nakagawa et al. (2004) have reported that SLO mediates the escape of intracellular *S. pyogenes* from endosomes into the cytoplasm. This has been shown by the lack of extrication of the *SLO* mutant strain from the endosomes in comparison to WT *S. pyogenes*. *S. pyogenes* present in the cytoplasm were shown to induce autophagic machinery and become enveloped by an autophagosome-like compartment which leads to intracellular killing of bacteria upon fusion of autophagosome with lysosomes. In contrast to this, it has been reported that SLO reduces both internalization of *S. pyogenes* into epithelial cells and intracellular killing by the lysosomes as shown by enhanced internalization of SLO deficient *S. pyogenes* into the cells via lysosomes with subsequent killing within the lysosomes. The SLO producing WT *S. pyogenes* however retards trafficking to lysosomes and thus may prolong the survival of bacteria by resisting intracellular lysosomal killing (Hakansson et al., 2005).

1.6 Objectives of the study

To understand the basic molecular mechanism of *S. pyogenes* pathogenesis that leads to the outcome of highly invasive human infections, it is essential to study the *S. pyogenes* virulence factors that contribute to enhance the virulence by evading the innate immune response of the host towards infection. The *S. pyogenes* serine protease ScpC is a novel virulence factor which impairs the recruitment of neutrophils at the site of infection by inactivating the host CXC chemokine IL-8, thereby enhancing the survival of *S. pyogenes* in the host. Studies have indicated that ScpC-mediated reduced local inflammatory response towards *S. pyogenes* infection significantly contributes to the enhanced virulence of *S. pyogenes* in a mouse model of soft tissue infection. Despite the important role of ScpC in enhancing *S. pyogenes* virulence, the biological characteristics of ScpC are undetermined and little is known about its biochemical properties. Thus, the first objective of this study was:

a) **To recombinantly express and characterize ScpC to further understand the contribution of ScpC in *S. pyogenes* pathogenesis of invasive soft tissue infections.**

Invasive deep tissue *S. pyogenes* infections are characterized by the accumulation of neutrophils in blood capillaries. However, neutrophils fail to transmigrate across the endothelium to the site of infection containing massive bacteria load. Vascular endothelial cells line the interior surface of blood vessels and act as a barrier between the blood and underlying tissue. The endothelium plays a central role in the inflammation process by secreting cytokines and regulating the transmigration of neutrophils via conveying signals from the inflammed tissue to the circulating neutrophils, to cause diapedesis. In non-inflammed tissue, endothelial cells control vessel wall permeability and quiesce circulating neutrophils. Recent studies have indicated the potential role of ScpC in retarding the transendothelial migration of neutrophils into the infection tissue. Based on these findings, it was hypothesized that breaching an endothelial barrier or interfering with endothelial cell function might in part contribute to the enhanced virulence of *S. pyogenes* during severe invasive soft tissue infections. Therefore, the second objective of this study was:

b) **To study the role of ScpC in the interaction of *S. pyogenes* with human vascular endothelial cells.**

2 Materials and Methods

2.1 *S. pyogenes* strains

Strain	Phenotype	Serotype
A475	Invasive isolate	M3
JS95	Muscle tissue isolate	M14 (Hidalgo-Grass et al., 2004)
KTL3	Blood isolate	M1
A416	Invasive isolate	M12
A471	Invasive isolate	M18

2.2 *E. coli* strains

Strain	Genotype	Reference
M15 [pREP4]	NaIS, StrS, RifS, Thi–, lac–, ara+, gal+, mtl–, F–, recA+, uvr+, lon+	Qiagen, 1994
DH5α	supE44, Δ(lac)U169 Φ80Δ(lacZ) M15, hsdR17, recA1, endA1, gyrA46, thi-1, relA1	Sambrook et al., 1989
SG13009	Lac- ara gal Mtl- F- recA+ uvr+	Qiagen, 1994

2.3 Vectors

Vector	Selection marker	Reference
pQE-60	Ampicillin (100 μg/ml)	Qiagen, 2003
pGEX-6P-1	Ampicillin (100 μg/ml)	Amersham, 2006
pJRS233	Spectinomycin (80 μg/ml)	(Perez-Casal et al., 1993)

2.4 Antibiotics

Antibiotic	Concentration	Company
Ampicillin	100 μg/ml	Applichem
Gentamicin	100 μg/ml	Sigma
Kanamycin	25 μg/ml and 250 μg/ml*	Applichem
Penicillin G	5 μg/ml	Sigma
Spectinomycin	80 μg/ml	Sigma

* For *S. pyogenes*

2.5 Chemical Reagents

Name	Company
2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)	Roche
Acetic Acid	Merck
Acetonitrile (HPLC-grade)	J.T. Baker
Agarose	Roth
Antarctic phosphatase	New England Biolabs
Ammoniumpersulfate (APS)	Roth
Ammoniumsulfate	J.T. Baker
N-propyl-gallate	Sigma
Bacto Agar	Difco
Bacto Peptone	Difco
Bacto Tryptone	Difco
Bacto Yeast Extract	Difco
2-mercaptoethanol	Sigma
Bovine serum albumine (BSA)	Applichem
Bradford reagent	Bio-Rad
Bromphenolblue	Merck
Calcium chloride ($CaCl_2$)	Merck
Chloroform	Baker
Citric acid	Roth
D-Glucose	Merck
Dipotassium hydrogen phosphate (K_2HPO4)	Merck
Disodium hydrogen phosphate (Na_2HPO4)	Merck
dNTP mix	Fermentas
Dithiothreitol (DTT)	Applichem
Ethanol	J. T. Baker
Ethidiumbromide	ICN-Biomedicals
Ethylene diaminetetraacetic acid (EDTA)	Applichem
Ferric nitrate ($FeNO_3$)	Fluka
Ferric sulfate ($FeSO_4$)	Merck
Fetal calf serum (FCS)	PAP
Fibrinogen (human)	Calbiochem
Formaldehyde	Fluka
Formamide	Roth
Gelatin	Sigma
Glycerol	Fluka
Glycine	Roth
Glycogen	Roche

Glutathione sepharose	Amersham
Glutathione (reduced)	Sigma
L-Glutamine	PAN Biotech
High fidelity polymerase	Roche
Hoechst	Molecular probes
Hydrochloric acid (HCl)	Fluka
Hydrogen peroxide	Applichem
Imidazole	Applichem
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Applichem
Lysozyme	Sigma
Magnesiumchloride ($MgCl_2$)	Merck
Methanol	Baker
Mowiol	Fluka
Mutanolysin	Fluka
Neopeptone	Difco
Ni-NTA sepharose	Qiagen
Papain	Sigma
Phenol	Applichem
Phenol/Chloroform/Isoamylalcohol	Applichem
PMSF	Applichem
Prolong Gold-antifade	Invitrogen
Pefabloc	Fluka
Ponceau S	Applichem
Potassium dihydrogenphosphate (KH_2PO4)	Roth
Prescission protease	GE-healthcare
Proteinase K	Fluka
Protein A sepharose	Pharmacia
Restriction enzymes	New England Biolabs /Fermentas
Saponin	Fluka
Sheep blood, defibrinated	Oxoid
Silver Nitrate	Merck
Skim milk	Difco
Sodium acetate ($NaCH_3COO$)	Merck
Sodium carbonate ($NaHCO_3$)	Merck
Sodium Chloride (NaCl)	Roth
Sodium dihydrogenphosphate (NaH_2PO4)	Roth
Sodium hydroxide (NaOH)	Merck
Sodiumdodecylsulfate (SDS)	Roth
Extravidin-HRP	Molecular Probes
Sucrose	Fluka
Sulfosalicylic acid	Merck

T4 DNA ligase	New England Biolabs
Taq-polymerase	Qiagen
TEMED	Roth
Trifluoroacetic acid	Applichem
Tris (Trizma base)	Sigma
Trypan Blue	Sigma
Trypsin	Sigma/Gibco
Tryptic Soy Broth	Beckton Dickinson
Todd-Hewitt Broth	Beckton Dickinson
Urea	J.T. Baker
X-Gal (5-Bromo-4-Chloro-3-indolyl-ß-D-galactopyranosid)	Applichem
Trypsin-EDTA	PAN Biotech
Coomassie Blue R-250	Serva
Triton X-100	Sigma
Human Interleukin -8	R&D
Dimetheylsulfoxide (DMSO) $(CH_3)_2SO$	Applichem
Latex polystyrene beads (1µm & 3µm)	Sigma
Tween 20	Roth
Acrylamid-Bisacrylamid (Rotiphorese Gel 30)	Roth

The chemicals which are not listed were either purchased from Sigma/Roche or referred to in the text.

2.6 Primers

Name	**Sequence 5' → 3'**
scpC-PR fwd *Bsp*HI	GCTAATTCATGACTGATGCGACTCAA
scpC-B/H rev *Bam*HI	TTCATTGGATCCGGTATTCACCTTTG
scpC-PR rev *Bam*HI	CCGCTGGATCCAGCTCCGTCAATATT
scpC-A rev *Bam*HI	CGGATCCTTGTGGTAGGTGATCTCCT
scpC-A fwd	TCATGACAGGAAAAGACAACTATGGC
scpC-A fwd *Bam*HI	CGGGATCCTATGTGACAGGAAAAGAC
scpC-A rev *Eco*RI	CCGGAATTCTCATGTTTGTGGTAGGTGATC
scpC B/H rev *Eco*RI	CGGGAATTCTCAGGTATTCACCTTTGTGTT
scpC-PR2 fwd	CTAATTCATGAAGGCACCGAAAGAAACT
pQE fwd	TTGTGAGCGGATAACAA
pQE rev	GTTCTGAGGTCATTACTGG
pQE fwd promoter Seq	CCCGAAAAGTGCCACCTG
pGEX fwd	GGGCTGGCAAGCCACGTTTGGTG
pGEX rev	CCGGGAGCTGCATGTGTCAGAGG

2.7 Antibodies

Antibodies	Conjugated with	Source/Company
Anti-human IL-8, monoclonal, hybridoma	--	R&D
Goat anti-human IL-8 IgG, polyclonal	--	R&D
Goat anti-mouse IgG	HRP	Sigma
Goat anti-rabbit IgG	Alexa Fluor® 488	Molecular Probes
Goat anti-rabbit IgG	Alexa Fluor® 568	Molecular Probes
Goat anti-rabbit IgG	HRP	Dianova
Mouse anti-human Integrin β 1	--	Chemicon
Mouse anti-human LAMP-1, clone H4A3, monoclonal	--	Beckton Dickinson
Mouse anti-human IL-8, monoclonal	Biotin	Abcam
Rabbit IgG	--	Sigma
Rabbit anti- *S. pyogenes*, polyclonal	--	Talay et al.
Rabbit anti-goat IgG	HRP	Dianova
Rabbit anti-mouse IgG	Alexa Fluor® 568	Molecular Probes
Rabbit anti-ScpC antibody, polyclonal	--	(this study)
Anti-EEA1, monoclonal	--	Beckton Dickinson

2.8 Cultivation of bacteria

2.8.1 Cultivation of *S. pyogenes*

S. pyogenes strains were grown overnight in either Todd-Hewitt broth supplemented with yeast extract (THY) at 37°C or in Tryptic Soy broth (TSB) under a humidified environment containing 5% CO_2. Antibiotics were added to the medium when required, and used as a selection marker for *S. pyogenes* mutant strains. To grow streptococci on solid media, bacteria were grown overnight at 37°C either on THY plates, which were made by supplementing 7 g of agar per liter of THY media. For plate culturing, *S. pyogenes* was grown on Columbia Blood Agar plates containing 5 % sheep blood (oxoid).

THY broth

30 g Todd-Hewitt Broth

10 g Yeast Extract

Dissolved in one liter of ddH_2O

Autoclaved for 15 min at 121°C

TSB

34.5 g Tryptic Soy Broth
Dissolved in one liter of ddH_2O
Autoclaved for 15 min at 121°C

2.8.2 Cultivation of *E. coli*

E. coli strains were grown in Luria Bertani (LB) media at 30°C or 37°C, while shaking. For the cultivation of *E. coli* M15 [pREP4] [pQE-60 *scpC*], ampicillin (100 µg/ml) and kanamycin (25 µg/ml) were added to LB media, as a selection marker. *E. coli* DH5α transformed with pGEX-6P-1 derivative were grown in LB media supplemented ampicillin (100 µg/ml). For plate culturing, LB agar plates were made by adding 7 g of agar per one litre of LB media.

LB media

10 g Bacto Tryptone
5 g Bacto Yeast Extract
10 g NaCl
Dissolved in one liter of ddH_2O, pH adjusted to 7.4 with NaOH.
Autoclaved for 15 min at 121°C

For long-term storage, *S. pyogenes* strains and *E. coli* strains were grown overnight in appropriate media and stationary phase bacteria were mixed with sterile glycerol in the ratio of 3:1 to give final concentration of 20% glycerol. The glycerol stocks were stored at -80°C in cyrotubes (Biozyme).

2.9 DNA isolation and quantification

2.9.1 Isolation of genomic DNA from *S. pyogenes*

S. pyogenes strains were grown overnight in THY media at 37°C and the DNA was isolated using the DNeasy DNA Isolation Kit (Qiagen) according to manufacturer's instructions. InstaGene™ Matrix (BioRad) was used alternatively for isolating the DNA for PCR analysis to check the presence of a gene in *S. pyogenes* strains. The colonies were gently scraped from the surface of agar plates and resuspended in 1 ml of dH_2O. The suspension was pelleted by centrifugation at 10,000 x *g*, for 1 min and the pellet was resuspended in 100 µl of InstaGene

matrix and incubated at 56°C for 20 min. The mixture was subsequently incubated at 100°C for 8 min. After vortexing for 10 sec, the sample was centrifuged at 10,000 x *g* for 3 min and the supernatant was used as template in PCR reactions.

2.9.2 Quantification of DNA

The DNA concentration was estimated by loading the equal volume of DNA sample and Gene Ruler DNA Ladder Mix (Fermentas) onto a 1% agarose gel. The amount of DNA was estimated in comparison to the DNA ladder according to manufacturer's instructions. Alternatively, the absorbance of DNA containing solution was measured using an UV-spectrophotometer and the concentration of DNA was calculated according to formula given below:

1 O.D. (260nm) = 50 mg DNA/ml

2.10 Polymerase chain reaction (PCR)

The DNA fragments were amplified by PCR using Taq polymerase and specific oligonucleotides complementary to the 5' end and the 3' end of the DNA fragment. The PCR for amplification of the gene of interest was carried out in three repetitive steps including denaturation, annealing and extension, using a thermocycler (Biometra). For all the cloning steps, the proof reading polymerase (Expand high fidelity PCR system) was used for the amplification of gene of interest, according to the manufacturer's recommendations. To verify the amplification of a gene fragment, samples were analysed by agarose gel electrophoresis as described in section 2.11 and the gel was visualized under UV light by using an UV transilluminator.

The following reagents were used for PCR reaction:

DNA (template)	10 – 100 ng
dNTPs (each 50 µM dNTP)	200 µM
Primers	0.2 µM
$MgCl_2$ (Qiagen)	250 µM
10 × Taq-Reaction buffer (Qiagen)	5 µl
Taq Polymerase (Qiagen)	0.5 µl

Added double distilled H_2O to make final volume of 50 µl.

PCR procedure for amplification of gene:

Step 1: 5 min 96°C

Step 2: 40 sec 96°C (denaturation)

Step 3: 40 sec 58°C (annealing)

Step 4: variable time 72°C (extension)

Step 2 - 4: repeated 29X

Step 5: 4 min 72°C

The length of the primers was chosen between 25-30 bp for the specific annealing of oligonucleotides with the gene of interest. The annealing of primers with template depends upon the melting temperature (Tm) of the oligonucleotides. The GC content of the sequence gives an indication of Tm of the primers. The Tm of primers was between 55-65°C. If the melting temperature of primers is too high, it can interfere with the function of Taq polymerase which has optimum activity at 75-80^{o}C. The Taq polymerase polymerizes approximately a thousand nucleotides per minute, therefore, duration of the extension step was chosen depending upon the size of the DNA fragment.

2.11 Agarose gel electrophoresis

DNA fragments were analysed by agarose gel electrophoresis by using agarose gels made by dissolving 1-2 % of agarose (w/v) in TAE buffer. The size of PCR products was determined by comparison with a GeneRuler^{TM} DNA Ladder Mix (Fermentas). The DNA samples was mixed with DNA loading dye (1:5) and loaded on an agarose gel. The gels were allowed to run at 80-120 V in TAE buffer for 45 min in a Minigel or Maxigel chamber (HorizonTM58). After electrophoresis, agarose gels were stained with ethidium bromide (1 µg/ml ethidium bromide in distilled H_2O), DNA was visualized under UV light at 254 nm using an UV transilluminator (Herolab) and the image of the gel was captured using a Herolab Enhanced Analysis System.

1 × TAE

40 mM Tris HCl (pH 7.9)

5 mM Sodium acetate

1 mM EDTA

5 × DNA Loading Dye

0.25 % Bromphenolblue (w/v)
0.25 % Xylencyanol (w/v)
30 % Glycerol (v/v)

2.12 Molecular cloning techniques

2.12.1 Digestion of DNA with restriction endonucleases

The DNA fragments (PCR amplicons or plasmids) were digested at specific sites using restriction endonucleases. Restriction endonucleases recognize specific regions with palindromic sequence and cut DNA at a specific site within this region, creating two single stranded complementary cut ends. For restriction digestion, DNA was incubated with restriction enzymes in an appropriate buffer according to the manufacturer's recommendations, and the reaction mixture was incubated at 37°C for 3-4 h. After the digestion of DNA with restriction enzymes, the enzymes were heat inactivated according to the manufacturer's recommendation. Alternatively, fragments were separated on a 1% agarose gel and the DNA band of the appropriate size was excised from the gel and DNA was eluted from agarose gel using the QIAquick Gel Extraction Kit (Qiagen). Alternatively, the reaction product was purified using the PCR Purification Kit (Qiagen) according to the manufacturer's recommendations.

2.12.2 Dephosphorylation of vector

To avoid religation of the vector, the 5' phosphate group of extended ends was dephosphorylated using antarctic phosphatase. For dephosphorylation, antarctic phosphatase reaction buffer (10X) was added in 1/10 volume to 1 µg of DNA (pre-digested with restriction endonuclease) along with 1µl (5 U) of antarctic phosphatase. The reaction mixture was incubated for 15 min at 37°C and the enzyme was inactivated by incubating at 65°C for 5 min.

2.12.3 Ligation of DNA fragments

The vectors and PCR amplicons digested with compatible restriction enzymes were mixed and the base pairing between overhangs generated by restriction digestion was further strengthened by covalently linking the 3'-hydroxyl group and 5'-phosphate group of the extended ends, using T4 DNA Ligase. 5-10 U of T4 DNA ligase was added to the reaction

mixture containing vector and insert DNA, mixed in a proportion dependent upon the size of vector and insert, to give a total reaction volume of 10 µl. The reaction mixture was incubated overnight at 16°C.

2.12.4 DNA precipitation

In order to increase the concentration of DNA in sample, DNA was precipitated by mixing the DNA containing sample with $1/10^{th}$ volume of 3 M sodium acetate (pH 4.6) and 2.5 fold volume of 98% ethanol. The reaction mixture was incubated overnight at -20°C, or alternatively at -80°C for 1 hour, centrifuged at 13,000 × *g*, 10 min at 4°C and the pellet was washed twice with 70% ethanol. The pellet containing precipitated DNA was air dried for 10-15 min and dissolved in TE buffer or distilled H_2O.

2.12.5 Preparation of chemically competent *E. coli* cells

The competent *E. coli* cells were prepared by a method as described by Dagert et al. (1979) with some modifications. A glycerol stock of the respective *E. coli* strain was thawed and added to 15 ml of LB broth and bacteria were allowed to grow overnight at 37°C while shaking at 120 rpm. The next day, the culture was diluted in the ratio of 1:15 in fresh LB broth and the absorbance of the culture was measured at 600 nm. Bacterial cells were grown to mid exponential phase (A_{600nm} 0.3-0.4) at 37°C, the culture was centrifuged at 5000 rpm for 10 min at 4°C and bacteria incubated for 10 min in 25 ml of 100 mM $MgCl_2$. After pelleting bacteria as described before, the cells were resuspended and incubated in 25 ml of 50 mM $CaCl_2$ at 4°C for 30 min. After centrifugation at 5000 rpm for 10 min, the pellet was resuspended in 5 ml of 50 mM $CaCl_2$ and glycerin solution, aliquoted, immediately frozen in liquid nitrogen and stored at -80°C.

2.12.6 Transformation of *E. coli* cells

For transformation, 100 µl of competent cells were thawed on ice and mixed with 10-20 ng of plasmid DNA or 5µl of ligation mixture and incubated on ice for 30 min to allow the binding of DNA to the cell wall. The cells were then heat shocked at 42°C for 90 sec in a waterbath and 1 ml of pre-warmed LB medium was added to the mixture. The cells were then incubated at 37°C for 1 h, while shaking at 120 rpm. The 100-500 µl of cell suspension was plated onto LB agar plates overlayed with the appropriate antibiotics and incubated overnight at 37°C. Resulting colonies were screened by using colony PCR (see section 2.12.7). The positive clones were inoculated in fresh pre-warmed LB medium containing appropriate antibiotics

and grown overnight at 37°C. The overnight culture of transformants was mixed with glycerol as described before and maintained as a glycerol stock at -80°C. In addition, the plasmid DNA was isolated using the Qiagen Plasmid Mini or Midi Preparation Kit according to the manufacturer's instructions and stored at -20°C.

2.12.7 Colony PCR

The *E. coli* transformants were analysed by colony PCR using primers specific for the gene of interest. For this, single bacterial colonies were picked using a sterile toothpick and resuspended in 13 µl of double distilled water. The bacterial cells were lysed by incubating at 96°C for 10 min. The PCR reaction was filled up with components as mentioned in 2.10, to give a final volume of 20 µl and amplified using a PCR program described in 2.10. The PCR products were analysed by agarose gel electrophoresis.

2.12.8 Colony Hybridization

The colonies were also characterized by colony hybridization, in order to select the positive clones. A nylon membrane (BioRad) was cut to the size of a petri dish and the circular membrane was overlayed on the surface of an agar plate. Three marks were spotted on the membrane and plate for orientation. Using a forcep and needle, the nylon membrane was carefully peeled from the agar surface and placed colony side up, onto a puddle of denaturing solution for 30 min. The membrane was then transferred to puddle of neutralizing solution for the next 30 min and briefly washed in 2 X SSC supplemented with 0.1% SDS. The membrane was dried and the DNA was cross-linked using a Stratalinker1800 (Stratagene) and hybridized with a DIG-labeled DNA probe. Hybridized DNA was detected as described by Sambrook et al. (1989).

2.12.9 DNA sequencing

The amount of DNA in the solution (purified PCR products or plasmid DNA) was calculated as described in 2.9.2. Approximately 100 ng of DNA, along with specific primers was prepared for sequencing. The DNA sequencing was conducted in the Department of Genome Analysis, Helmholtz Centre for Infection Research. The analysis of sequence data was performed using Chromas 1.44 and DNAMAN. The Oligonucleotides were ordered at MWG Biotech and diluted with distilled water to a concentration of 100 pM/µl.

2.13 Recombinant protein expression in *Escherichia coli*

2.13.1 Construction of plasmids for cloning and overexpression of protein

For expression of recombinant ScpC (rScpC) with 6xHistidine as an affinity tag, *scpC* encompassing 642-5018 nucleotides (accession no. DQ192030), excluding pre-pro domain and cell wall-anchoring domain (full-length *scpC*) was amplified with PCR using DNA of *S. pyogenes* invasive strain A416. The proof-reading polymerase (Expand High Fidelity PCR system) was used according to the manufacturer's instructions. The oligonucleotides (scpC-PR fwd *Bsp*HI and scpC-B/H rev *Bam*HI) were designed with restriction sites, in order to amplify and incorporate the restriction site into 1458 aa fragment corresponding to 106-1564 amino acid of ScpC (ABA33824.1). The amplicon was then digested with restriction enzymes *Bsp*HI *and Bam*HI as described in 2.12.1. The digested fragment was ligated into pQE-60 vector pre-digested with restriction enzyme *Nco*I (compatible with *Bsp*H1) and *Bam*HI using T4 DNA ligase (Qiagen).

The pQE-60 derivative containing *scpC* was then transformed into the competent *E. coli* strain M15 containing repressor plasmid pREP4 as described in 2.12.6. pQE-60 contains β-lactamase gene (*bla*) as a selection marker which confers resistance to ampicillin at concentration of 100 μg/ml and pREP4 confers resistance to kanamycin at concentration of 25 μg/ml. Therefore, the positive clones, M15 [pREP4] [pQE60-*scpC*] were selected on LB agar plates containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The presence of the insert gene (*scpC*) was checked by colony PCR using primers specific for *scpC*. Expression of ScpC by positive clones was checked on a small scale by running the samples on SDS-PAGE gel after incubating the 10 ml of *E. coli* cell culture with IPTG.

2.13.2 Heterologous expression and purification of recombinant protein

The transformants selected were grown overnight in LB medium containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The overnight cultures were mixed with glycerol to give a final concentration of 20% glycerol and stored at -80^{o}C in cryotubes. The 5 ml of overnight cultures were added to 1 litre of LB broth supplemented with the appropriate antibiotics and incubated at 37^{o}C until an absorbance (A_{600nm}) was reached at 0.3. The expression of recombinant protein encoded by pQE-60 is rapidly induced by the addition of isopropyl-β–D-thiogalactoside (IPTG). IPTG binds to the lac repressor protein rendering it incapable of binding the operator region. Once repressor protein is inactivated, *E. coli* RNA polymerase binds to promoter region and transcribes the insert sequences present downstream to the T5

promoter region which is then translated into rScpC. The *E. coli* cells grown to mid-exponential phase were then incubated with 2 mM IPTG for 4 h at 37°C to induce expression of recombinant protein and then harvested by centrifugation at 6000 × *g* for 15 min at 4°C. The pellet was resuspended in 10 ml of lysis buffer containing 1 mM PMSF. The *E. coli* cells were lysed using a French Press (SLM Aminco, SLM Instruments, 1000 bar). The cell debris were removed by centrifugation at 10,000 × *g* for 20 min at 4°C and the clear lysate was obtained. Four millilitres of 50% Nickel–nitrilotriacetic acid (Ni–NTA) resin slurry was added to the bacterial cell lysate and incubated for 1 h at 4°C while rotating, to allow the protein to bind to the resin. This resin with immobilized protein was then packed into a column. After the column was washed with wash buffer containing 0.02 M imidazole, the recombinant protein was eluted with 0.25 M imidazole (elution buffer). 6xHis tagged recombinant protein was purified from bacterial cell lysate using Ni-NTA agarose under native conditions, according to the manufacturer's instructions. The protein purification was performed with either batch or column method. Fractions containing the protein were pooled, dialysed against PBS overnight and stored at -20°C. All purified protein fractions were analysed by SDS-PAGE and protein concentration was determined using a Bradford assay and/or by using the UV spectrophotometer as described below in section 2.14.4.

PBS (10X)

160 g NaCl
4 g KCl
15.2 g Na_2HPO_4
4 g KH_2PO_4
Added to 1.8 l of distilled H_2O
pH was adjusted to 7.4 with NaOH

Lysis buffer

6.9 g NaH_2PO_4. H_2O
17.54 g NaCl
0.68 g imidazole
Added to 1 l distilled H_2O, pH was adjusted to 8.0 using NaOH

Wash buffer

6.9 g NaH_2PO_4. H_2O
17.54 g NaCl
1.36 g imidazole

Added to 1 l distilled H_2O , pH was adjusted to 8.0 using NaOH

Elution buffer

6.9 g NaH_2PO_4. H_2O

17.54 g NaCl

17.00 g imidazole

Added to 1 l distilled H_2O , pH was adjusted to 8.0 using NaOH

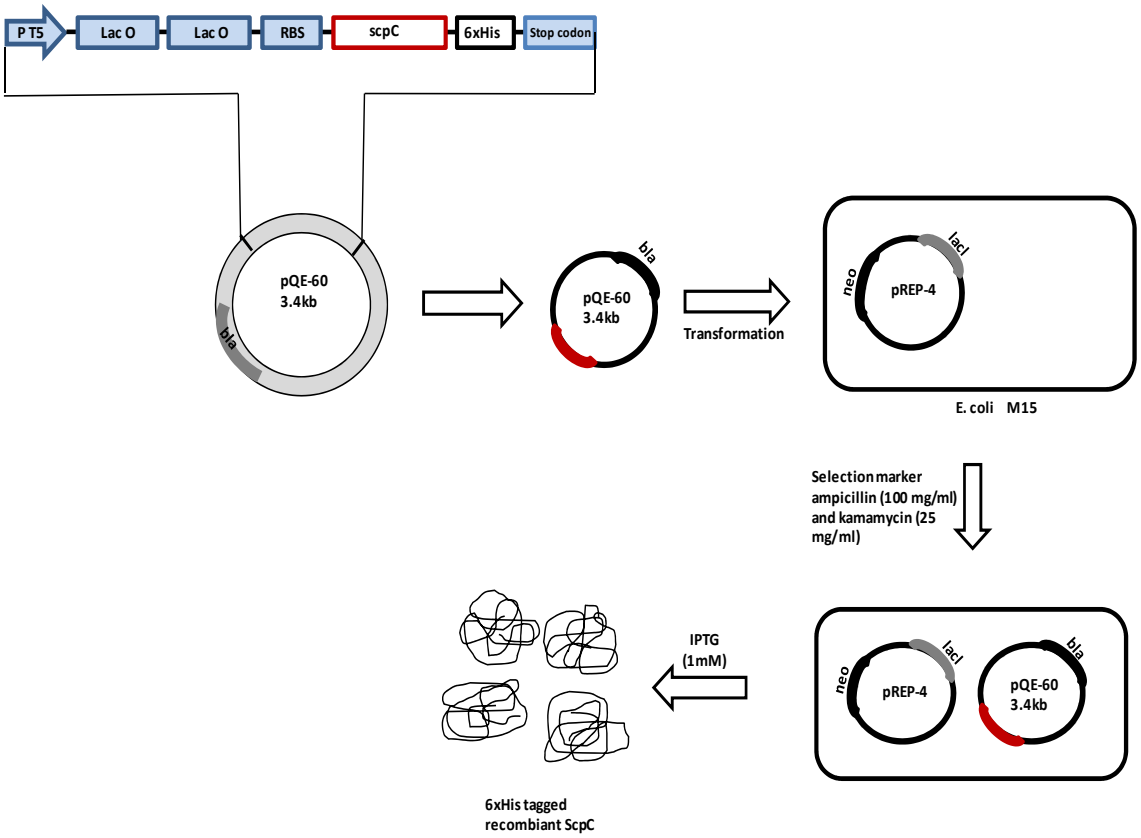


Figure 6. Schematic overview of cloning and recombinant expression of ScpC. Full-length *scpC* was expressed using pQE-60 expression vector. Expression in pQE-60 is under the control of T5 promoter and operator element. For the expression of rScpC, pQE-60 derivative containing *scpC* was transformed into competent *E. coli* strain M15 containing pREP4 repression plasmid.

2.13.3 Recombinant expression of fusion proteins encompassing subfragments of ScpC

Recombinant expression and purification of a fusion proteins encompassing the N-terminal PR-domain of ScpC, spanning 106-689 aa (EC-PR, accession no. ABA33824.1), and a fusion protein encompassing the PR along with the A-domain, spanning 106-1126 aa (EC-PR+A) were performed as described above in section 2.13.2.

2.13.4 Cloning, expression and purification of fusion proteins with GST tag

The C-terminal domains of ScpC, the A-domain spanning 690-1127 aa (EC-A, accession no. ABA33824.1) and the A-domain along with the B/H-domain, spanning 690-1560 aa (EC-A+B/H), were expressed as recombinant fusion proteins tagged with glutathione S-transferase (GST). The DNA fragments were amplified with primers (scpC-A fwd *Bam*HI, scpC-B/H rev *Eco*RI and scpC-A rev *Eco*RI) designed to incorporate restriction site and stop codon and cloned into pGEX-6P-1 as described in 2.13.1**.** The *E. coli* strain DH5α was transformed with pGEX-6P-1 derivative containing insert. The transformants were selected on LB agar plates containing ampicillin (100 μg/ml). The positive clones were grown overnight in LB medium containing ampicillin (100 μg/ml). The next day, 5 ml of overnight grown culture was added to fresh 1 liter of LB medium and incubated at 37^{o}C in a shaking incubator (120 rpm) and bacteria was grown to A_{600} 0.3. Afterwards, the expression of recombinant protein was induced by addition of 2 mM IPTG for 4 h at 37°C and the culture was centrifuged at 6000 × *g* for 15 min at 4°C. The pellet was resuspended in 10 ml of PBS buffer containing 1 mM PMSF (serine protease inhibitor) and lysozyme (1mg/ml). The cells were lysed using a French Press (SLM Aminco, SLM Instruments, 1000 bar) and cell debris was removed by centrifugation at 10,000 × *g* for 20 min at 4°C. The clear lysate was transferred to a falcon tube, incubated with glutathione sepharose, and protein was eluted under mild non-denaturing conditions according to manufacturer's instructions. The positive clones were stored as glycerol stocks at -80^{o}C. The plasmid DNA was isolated from 2 ml of overnight culture and sequence of the insert was verified by sequencing**.**

Elution buffer

50 mM Tris-HCl
10 mM reduced glutathione
pH was adjusted to 8.0

Prescission cleavage buffer

50mM Tis-HCl
150mM NaCl
1mM EDTA
1mM DTT
pH was adjusted to 7.5

2.14 Protein analysis

2.14.1 SDS-PAGE

In sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), migration of protein is determined by the molecular weight of the polypeptide (Laemmli et al., 1970). SDS is an anionic detergent which denaturates proteins by wrapping the hydrophobic tail around the polypeptide backbone. For almost all proteins, SDS binds at a ratio of approximately 1.4 g SDS per gram of protein, thus conferring a net negative charge to the polypeptide in proportion to its length. SDS also disrupts hydrogen bonds and unfolds the protein molecules thus minimizing differences in molecular form by eliminating the tertiary and secondary structures. The proteins can be totally unfolded by using a reducing agent such as β-mercaptoethanol, which cleaves the disulfide bonds between cysteine residues.

For SDS-PAGE analysis of the protein samples, resolving gels containing 8%-17.5% acrylamide were prepared. A stacking gel containing 5% acrylamide was used for all the SDS-PAGE gels. The protein samples were mixed with sample loading buffer in a ratio of 2:1 and boiled for 5 min. The samples were loaded on gel and electrophoresed using Mini-Protean 3 cell (BioRad) by applying 150 V (constant voltage). A PageRuler^{TM} Prestained Protein Ladder (Fermentas) was loaded as a standard for determining the size of protein in comparison to the ladder mix. Following electrophoresis, the gel was either stained with Coomassie Brilliant Blue R-250 or silver nitrate (2.16.1) or used for western blot analysis as describedin section 2.14.2.

<u>SDS-Loading buffer</u>

0.2 M Tris-Cl (pH 6.8)
β -Mercaptoethanol
3 % SDS
30 % Glycerol
0.2 % Bromphenolblue

<u>Resolving Gel</u>

10 – 15 % Acrylamide/Bisacrylamide (30:0.8)
0.37 M Tris/HCl (pH 8.8)
0.1 % SDS
0.5 µl/ml TEMED
0.5 mg/ml APS

Stacking Gel

5 % Acrylamide/Bisacrylamide (30:0.8)
0.125 M Tris HCl pH 8.8
0.1 % SDS
0.5 µl/ml TEMED
0.5 mg/ml APS

5 × Running buffer

30 g Tris
144 g Glycine
10 g SDS
pH adjusted to 8.6
Added to 2 l of dH_2O

2.14.2 Western blotting

For western blot analysis, proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (BioRad) in blotting buffer at 35 V for 2 h at 4°C using a Mini Trans-Blot cell (BioRad). Following this, protein transfer was confirmed by staining the nitrocellulose membrane with Ponceau as described in section 2.16.3. After washing the membrane with PBST (PBS supplemented with 0.05% Tween 20), the membrane was incubated overnight with blocking buffer (5% skim milk dissolved in PBST) at 4^oC to block the non-specific binding sites on the membrane. To check the expression of recombinant full-length ScpC and fusion protein constructs of ScpC subfragments, the membrane was probed with rabbit polyclonal anti-ScpC IgG, diluted in 1:1000 ratio in PBST, for 1 h at RT. The membrane was washed twice with PBST and incubated with anti-rabbit IgG (diluted 1:2000 in PBST) conjugated with horseradish peroxidase (HRP) for 1 h. The blot was washed three times with PBST for 15 min, and immunoreactive protein was detected by using the ECL Western Blotting Detection Kit (Amersham).

Transfer Buffer

50 mM Tris-Base
40 mM Glycine
20 % Methanol (v/v)
Added to ddH_2O

Blocking Buffer

5 % (w/v) Skim milk or 3 % BSA
Dissolved in PBST

2.14.3 Dot blot

The interaction of recombinant protein with the ligand was analysed by dot blotting**.** For this, samples containing 2-4 µg of recombinant protein were applied directly onto nitrocellulose membrane and the membrane was dried for short time at room temperature. To differentiate between nonspecific and positive signals, both 2-4 µg of purified GST and 2 µl of a cell extract of the host strain without plasmid was applied to the membrane. Non-specific protein binding was inhibited by incubating the membrane in blocking buffer (5% skim milk dissolved in PBST) for 1 h at RT, membrane was probed and immunodetection was performed as mentioned in section 2.14.2.

2.14.4 Determination of protein concentration

The concentration of purified proteins was determined by a method described by Bradford et al. (1976). For this, 40 µl of Bradford solution (BioRad) was added to 10 µl of a protein solution in a microtiter plate, and double distilled water was added to give a total volume of 200 µl. The absorption at 595 nm was measured by using an ELISA-Reader (Tecan Sunrise) without any prior incubation. The standard curve was plotted by using BSA as a standard. The absorbance of known concentrations of BSA was measured at 595 nm and the amount of protein in the solution was estimated by comparing with the standard. Alternatively, the concentration of protein in solution was determined by using an UV-spectrophotometer. The spectrophotometer (UV deuterium lamp) was turned on 15 min before measuring the absorbance of the sample. The absorbance of the protein solution was measured at 280 nm in a quartz cuvette. To eliminate absorbance due to possible nucleic acid contamination, absorbance of the solution was also measured at 260 nm. The buffer solution was used as reference and the concentration of protein in solution was determined using a formula given below:

Concentration (mg/ml) = (1.55 x A280) – (0.76 x A260) (Waddell, 1956)

2.14.5 Peptide sequencing

NH_2-terminal amino acid sequence of the purified recombinant protein was determined by Edman degradation. Briefly, about 10 µg of purified protein was resolved by SDS-PAGE and electrotransferred onto a polyvinylidine fluoride membrane (Bio-Rad) preactivated in 100% methanol, at 20 V for 2 h at 4°C. The protein was visualized by staining with 0.1% coomassie in 50% methanol. The band of interest was excised from the membrane and destained in 50% methanol and finally washed in double distilled H_2O. The amino acid sequence of the sample was identified by Edman degradation performed using an Applied Biosystems 494 protein sequencer at the Department of Biophysical Analysis, Helmholtz Centre for Infection Research, Braunschweig.

2.15 Generation of anti-ScpC polyclonal antibodies and purification of IgG

To raise specific antibodies against recombinant full-length ScpC, 500 µg of purified recombinant ScpC was sent to Pineda Antikorper-service (Berlin) for rabbit immunization. Rabbit were immunized four times with 100 µg of recombinant ScpC per dose at one week interval and serum was collected 2 weeks after the final immunization. For purification of anti-ScpC IgG antibodies from serum, a column was packed with protein-A speharose CL-4B and equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). Following the equilibration step, 2 ml of rabbit immune serum was applied to a column. Afterwards, the column was washed with PBS and bound IgG were eluted using 0.1 M glycine/HCl (pH 3.0). The elutant was neutralized by adding 50 µl of Tris-HCl (pH 8.0) to 1 ml elutant and the IgG fractions were analysed by SDS-PAGE. The IgG containing fractions were pooled, dialysed against PBS for 16 h and stored at -20°C. The concentration of purified IgG was measured using an UV-spectrophotometer (Pharmacia Ultrospec III) as described in section 2.14.4.

Elution buffer

0.1 M Glycin (pH 3.0)

Neutralizing buffer

1.5 M Tris-HCl (pH 8.8)

2.16 Staining methods

2.16.1 Sliver staining

Silver staining is the most sensitive method for the permanent visible staining of proteins in polyacrylamide gels with protein detection limit in the nanogram range. It is 30-50 fold more sensitive than Coomassie Brilliant Blue R-250 dye. To determine IL-8 degradation, 10 µl of culture supernatant of bacterial strains was co-incubated with equal volume of human IL-8 (5µg/ml) overnight at 37^{o}C and electrophoresed on SDS-PAGE gel (17.5%). For silver staining, the gel was impregnated with soluble silver ions and developed by treating with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. Staining of gel was performed using the Silver Stain Plus kit (BioRad). The procedure was followed according to manufacturer's recommendations.

2.16.2 Coomassie staining

To visualize proteins on the SDS-PAGE gel, the gel was stained for 2-3 h in Coomassie Brilliant Blue R-250 staining solution. The gel was then washed with water and incubated in destainer solution for 2-3 h or alternatively kept overnight in destainer. Gels were scanned using a HP DeskScan II 6100C/T and anlysed using GIMP 2.6.1.

Coomassie stain solution

0.25 % (v/v) Coomassie Brilliant Blue R-250
50 % (v/v) Methanol
10 % (v/v) Acetic acid
Added to distilled water

Destainer

20 % (v/v) Methanol
7 % (v/v) Acetic acid
Added to distilled water

2.16.3 Ponceau staining

To confirm the successful transfer of proteins from SDS-PAGE gel onto a nitrocellulose membrane during western blotting, the membrane was stained with Ponceau S stain for 1-2 min. The staining of the membrane with Ponceau S allows the reversible staining of proteins on a nitrocellulose membrane. The membrane was then rinsed with dH_2O and the bands were

examined. Afterwards, the membrane was washed several times with PBST until the bands were not visible and probed for western blot analysis as described in section 2.14.2.

Ponceau stain solution

0.2 % (v/v) Ponceau S
3 % (v/v) Trichloroacetic acid
3 % (v/v) Sulfosalicylic acid

2.17 Immunolabeling methods and Electron microscopy

2.17.1 Immunolabeling of cell surface-bound ScpC

To characterize the surface localization of ScpC, *S. pyogenes* strains were grown overnight in THY medium. The overnight cultures were diluted in 1:15 ratio in fresh THY medium and the absorbance was measured at 600 nm. The *S. pyogenes* strains were grown at 37^{o}C to mid exponential phase (A_{600nm} 0.3-0.4). For labeling, 2-3 ml of early-exponential phase *S. pyogenes* cell culture was centrifuged at 4000 rpm for 10 min and the pellet was washed two times with PBS. The bacterial cells were incubated with rabbit anti-ScpC polyclonal antibody diluted 1:100 in PBS supplemented with 10% fetal calf serum for 1 h at room temperature, while shaking at 70 rpm. The cell suspension was centrifuged at 4000 rpm for 10 min and the pellet was washed twice with PBS to remove unbound antibody. The pellet was resuspended in goat anti-rabbit Alexa Fluor® 488 conjugated secondary antibody, diluted 1:200 in PBS supplemented with 10 % fetal calf serum, for 1 h in the dark at room temperature. As a negative control, the anti-ScpC antibody was omitted in one series of experiments. The suspension was centrifuged at 4000 rpm for 10 min. The bacterial cells were then washed two times with PBS and the pellet was resuspended in 50 µl of PBS. 10 µl of each suspension was placed onto a slide and fixed with 10 µl of Mowiol and analyzed by fluorescence microscopy using a Zeiss Axiophot microscope and images were captured using Zeiss AxioCam HRc 14-bit color CCD camera**.**

To check the reassociation of ScpC to the surface of *S. pyogenes***,** *S. pyogenes* strains were grown overnight in THY medium. The antibiotics were added to the medium in appropriate concentration. The next day, strains were sub-cultured as described above and grown to early exponential phase (A_{600} 0.3-0.4). The *S. pyogenes* cultures were centrifuged at 4000 rpm for 10 min. The supernatant of WT JS95 was transferred to a 15 ml falcon tube and sterile filtered. The pellet of JS95 Δ*scpC/*Δ*scpA* and A475 Δ*scpC* mutant were incubated with the

supernatant of WT JS95 for 1 h at 37°C. After the incubation period, the bacterial cells were washed twice with PBS, probed with anti-ScpC antibody and anti-rabbit Alexa Fluor® 488 conjugated secondary antibody, mounted on glass slides and analysed by fluorescence microscopy as described above.

2.17.2 Immunogold labeling to identify cell surface-associated ScpC

For immunogold labeling of surface-bound ScpC, *S. pyogenes* strains were grown overnight in THY medium. The next morning, each strain was subcultured by diluting in 1:100 ratio in fresh THY medium and grown to mid exponential phase (A_{600} 0.3-0.4), samples were fixed with 1% formaldehyde for 1 h on ice and washed three times with cacodylate buffer containing 10 mM glycine for quenching free aldehyde groups. One millilitre of the bacterial suspension was centrifuged and pellet was resuspended in 180 µl PBS. To this solution, 20 µl of protein A purified anti-ScpC IgG (3.5 mg/ml) were added and incubated for 1 h at 30°C. After centrifuging the bacterial suspension at 4500 x*g*, bacterial cells were washed three times with PBS and resuspended in 190 µl of PBS. The suspension was then incubated with 10 µl of colloidal protein A coated gold particles (15 nm) solution (British BioCell) for 30 min at 30°C. The bacterial suspension was centrifuged at 4500 x*g* for 10 min at room temperature. The cells were washed three times with PBS and fixed with 2% glutaraldehyde for 10 min at room temperature. The fixed cells (25 µl) were dropped onto the coverslips and allowed to attach for 10 min at RT and transferred to a 24 well plate containing 800 µl of a fixing solution. The Δ*scpC* deletion mutant of M3 *S. pyogenes* strain A475 was constructed in our laboratory by Dr. Dorothea Zahner (2002; Helmholtz Centre for Infection Research, Braunschweig).

2.17.3 Field-emission scanning electron microscopy (FESEM)

For FESEM analysis, the coverslips were washed twice with TE buffer and dehydrated on ice by incubation in a raising series of acetone (10, 30, 50, 70, 90, 100%) with each step for 15 min. The samples were warmed to RT and critical-point dried using liquid CO_2 (CPD030, Balzers). For detection of gold-particles, the coverslips were then fixed on aluminium-plates and covered with a thin layer of coal (MED020, Balzers). The samples were analyzed with a Zeiss field emission scanning electron microscope (DSM982 Gemini) at 5 kV using the Everhart-Thornley SE detector and the inlens secondary electron (SE) detector in a 50:50 ratio.

Cacodylate Buffer

100 mM Cacodylate (pH 6.9)
90 mM Sucrose
10 mM $MgCl_2$
10 mM $CaCl_2$

Fixative

2% (w/v) Glutaraldehyde
5% (w/v) Formaldehyde
in Cacodylate Buffer

TE buffer

10 mM Tris
2 mM EDTA, pH 6.9

2.18 Enzyme-linked immunosorbent assay (ELISA)

S. pyogenes strains were grown overnight in THY medium. The next day, the culture was diluted in 1:15 ratio in fresh pre-warmed THY medium and grown to mid-exponential phase by incubating at 37°C. Antibiotics were added to the medium for growing the A475 Δ*scpC* (spectinomycin, 80 µg/ml), JS95 Δ*scpC*/Δ*scpA* (kanamycin, 250 µg/ml and spectinomycin, 50 µg/ml), JS95 Δ*scpA* (spectinomycin, 50 µg/ml) mutant strains. The bacterial culture was centrifuged at 4500 rpm for 10 min and the pellet was washed twice with PBS to remove the remaining yeast extract. The pellet was then resuspended in Dulbecco's Modified Essential Medium (DMEM; PAA Laboratories) supplemented with 0.1% fetal calf serum and incubated overnight at 37°C. For growing Δ*scpC* mutant strains, the appropriate antibiotics were added to DMEM. On the next day, the culture was centrifuged at 4500 rpm for 8 min and the supernatant was transferred to a 15 ml falcon tube and filtered using a filter with 0.2 µm pore size. The supernatant was diluted in a 1:1 ratio in PBS and 10 ng of IL-8 was added to give a final volume of 100 µl. For testing the activity of recombinant proteins, approximately 5-20 µg of recombinant protein was added to 10 ng of recombinant IL-8, and PBS was added to give a final volume of 100 µl. The reaction mixture was incubated for 16-18 h at 37°C. The amount of residual interleukin-8 was measured by ELISA using human IL-8 Quantikine ELISA kit (R&D) in accordance with the manufacturer's instructions. The absorption was measured at 450 nm by using ELISA-Reader (Tecan Sunrise).

For inhibition assay, culture supernatant of *S. pyogenes* and rScpC was incubated with affinity-purified anti-ScpC IgG (50 µg/ml) for 1 h at RT and then co-incubated with recombinant IL-8 at 37°C for 16 h. The residual IL-8 was measured by ELISA as described above. The *scpC* deletion mutant of M14 serotype *S. pyogenes* invasive strain JS95 (JS95 Δ*scpC*/Δ*scpA*) was kindly provided by Prof. Emanuel Hanski (The Hebrew University-Hadassah Medical School, Jerusalem, Israel).

2.19 Ligand overlay assay

For ligand overlay assay, recombinant fusion proteins (10 µg of each protein) encompassing full-length ScpC, N-terminal domains of ScpC (EC-PR, EC-PR+A), C-terminal domains of ScpC (EC-A+B/H) and cell lysate of *E. coli* M15 containing pQE-60 derivative with the A-domain insert were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane (see section 2.14.2). Alternatively, for the dot blot analysis, 5 µg of each recombinant protein was directly loaded on the nitrocellulose membrane and the membrane was dried briefly. Purified recombinant GST (5 µg) was used as a negative control. 10 µg of IL-8 was radiolabeled with ^{125}I using the chloramine-T method as described by Hunter and Greenwood (1962). The membrane was blocked for 30 min with 5% skim milk in PBS at RT and incubated with 6 µg [^{125}I] IL-8 dissolved in 15 ml of PBST ($4x10^5$ c.p.m./ml) for 1 h while rotating at room temperature. After extensive washing (5-7 times with PBST), the interaction of immobilized recombinant protein with radiolabeled IL-8 was detected by exposing an X-ray film (Kodak BioMax XAR) overnight to the membrane and the X-ray film was developed next day.

2.20 Flow cytometry

For the detection of ScpC on the surface of *S. pyogenes* strain JS95, bacteria were grown overnight in THY medium. The next day, culture was diluted 1:15 in THY medium and bacteria were incubated at 37^{o}C. Bacterial cells were collected at different growth phases (mid-exponential phase; A_{600nm} 0.4 and late exponential phase; A_{600nm} 0.85) by centrifugation at 4000 rpm for 8 min and washed twice with PBS. The cells were then incubated for 1 h with rabbit polyclonal anti-ScpC antibody (1:100 in PBS supplemented with 10% FCS) and anti-rabbit fluor® 488 conjugated antibody. The fluorescence intensity of each sample was

measured using a FACS calibur cytometer (Becton & Dickinson) and data were analyzed with the Cellquest software.

2.21 Peptide analysis

2.21.1 Synthesis of chromogenic substrate

The synthesis of para-nitroanilide peptides were performed as described previously by Abbenante et al. (2001). A chromogenic substrate with the peptide sequence, Ac-K-E-N-W-V-Q-pNA and a control peptide Ac-N-E-K-Q-V-W-pNA were synthesized. Briefly, 2 g chlorotrityl-chloride resin was treated with phenylene diamine (2 mM) and diisopropylethyl amine (2 mM) in DMF at room temperature for 24 h. After washing with dimethylformamide (DMF) and dichloromethane (DCM) the resin was dried and 515 mg of the dry material was treated with Fmoc-Gln (Trt)-OH (800 µM), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU, 800 µM) and di-isopropylethyl amine (1.6 mM) in DMF for 2 h at RT. After washing the resin with DMF, the Fmoc group was cleaved by 10 min treatment with 20% piperidine in DMF. The remaining amino acids of the peptide sequence were coupled in the same way as described above. After the final acetylation, the resin was washed with DMF and DCM. The dry resin was treated for 5 h repeatedly with 2% trifluoro acetic acid in DCM. The filtrate was collected and dried by evaporation. The residue was taken up in 110 ml acetonitrile and 100 ml H_2O were added. After the addition of Oxone (2 g, 3 mM), the mixture was incubated for 18 h at RT, while shaking. After filtering off the Oxone, the solvents were evaporated and the crude product was purified by preparative high performance liquid chromatography (HPLC) and characterized by analytical HPLC and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

2.21.2 Synthesis of 16-mer peptide

The solid-phase synthesis of the peptide (Ac-^{52}DPKENWVQRVVEKFLK^{67}-amide) was carried using a Pioneer Automatic Peptide Synthesizer (Applied Biosystems) employing Fmoc chemistry with TBTU activation as described by Reid and Simpson et al. (1992) with some modifications. Side chains were protected and peptide was synthesized using coupling time of 1 h**.** The peptide was cleaved from the resin and deprotected by 3 h treatment with TFA containing 3% triisobutylsilane and 2% water (10 ml/g resin). The resin was removed by filtration and washed several times with acetic acid. The combined filtrate and washings were dried by evaporation. The crude peptides were dissolved in water/acetonitrile in 1:1 ratio and passed over 6 ml octadecyl disposable extraction columns. After rinsing the columns with

acetonitrile, most of the acetonitrile of the combined eluents was evaporated. After precipitation with t-butyl methylether, samples were centrifuged. The samples were lyophilized and the resulting crude peptide was purified by preparative HPLC (RP-18) with a water/acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA).

2.21.3 Analysis of substrate specificity of protease

The enzymatic activity of both rScpC (15-20 µg) and *S. pyogenes* culture supernatant against synthetic chromogenic substrate was assayed in a microtitre plate by measuring the release of para-nitroaniline from para-nitroanilide derived peptide via measuring the absorbance changes at 405 nm at various time intervals. The enzymatic activity of rScpC against the peptide substrates was tested at substrate concentrations of 0.5-1 mM by incubating for 10 min-1 h at different temperatures (25°C, 30 °C, 37 °C) in 0.1 M PBS buffer (pH 7.0) or Tris-HCl buffer with or without the presence of 2-5 mM Ca^{2+}.

For analysing the activity of rScpC against the 16-mer synthetic peptide substrate, peptide (0.1 mM) was incubated with rScpC (20 µg) for overnight at 37°C in PBS. Each digest was separated by reversed-phase HPLC using a linear gradient from 0 to 30% acetonitrile in 0.1%TFA and average molecular mass of peptide fragments was determined by MALDI-MS. The synthesis of peptides was kindly performed by Dr. Werner Tegge (Dept. of Chemical Biology, Helmholtz Centre for Infection Research, Braunschweig).

2.22 Cell culture methods

2.22.1 Cultivation of human endothelial cells

Primary human umbilical vein endothelial cells (HUVEC; PromoCell) were aliquoted and cryopreserved in liquid nitrogen. Endothelial cell growth medium 2 (EGM 2, PromoCell) was mixed with supplement mix (PromoCell) and penicillin/streptomycin. The pre-warmed medium (12 ml) was added to gelatin coated petri dishes with a 10 cm diameter. The cells were thawed on ice, resuspended in 200 µl of medium and added to the cell culture dish containing pre-warmed medium. HUVEC were cultivated for 2-3 days at 37°C in humidified environment under 5% CO_2 conditions in an incubator (Forma Scientific), until cells formed a confluent monolayer. All the cell culture experiments with HUVEC were performed with cells at 2^{nd}-4^{th} passage. The cells were grown to confluency and sub-cultured at an interval of 2-3 days. The monolayer was washed with 5 ml of wash buffer (HEPES BSS buffer) and the cells were incubated with 3 ml of trypsin EDTA buffer (PromoCell) to detach them from the

surface of the cell culture plate followed by the addition of 7 ml of pre-warmed fresh medium. The cells were resuspended, transferred to a falcon tube and centrifuged at 900 x*g* for 10 minutes. The supernatant was discarded and the pellet was resuspended in EGM 2 supplemented with growth factors and penicillin/streptomycin. 0.5-1 ml of cell suspension was added to cell culture dishes (10 cm diameter) containing 12 ml of pre-warmed medium and incubated at 37^{o}C in a humidified environment containing 5% CO_2. For microscopy studies, glass cover slips with a diameter of 12 mm (Nunc) were sterilized in a covered dish at 180^{o}C in a dry autoclave. To enhance the attachment of HUVEC, sterile coverslips were coated with 0.1% gelatin dissolved in double distilled water. For this purpose, 200 µl of gelatin solution was added to each well and incubated for 15 min at RT and then removed prior to the addition of cells into each well.

2.22.2 *In vitro* infection assay of HUVEC with *S. pyogenes*

To study the interaction of *S. pyogenes* or rScpC with HUVEC, the monolayer was washed with 5 ml of washing buffer (HEPES BSS buffer). The monolayer was incubated with 3 ml of trypsin EDTA buffer and resuspended in 7 ml of pre-warmed fresh medium. The cell suspension was centrifuged at 900 xg for 10 minutes. The supernatant was removed and cells were diluted in EGM 2 supplemented with growth factors and penicillin/streptomycin to give a final concentration of approx. 1 x 10^5 cells/ml. 500 µl of cell suspension was seeded into a 24 well cell culture plate to give a semiconfluent monolayer after 24 h. On the day of infection, cells were washed twice with endothelial cell basal medium without antibiotics and 500 µl of fresh medium was added into each well and kept at 37^{o}C in humidified environment containing 5 % CO_2 in an incubator before infecting the cells with *S. pyogenes*.

2.22.2.1 Preparation of inoculum and infection assay

Bacteria were grown overnight by gently scraping the colonies from the surface of blood agar plates with an inoculation loop or by inoculating with 50 µl of glycerol stock in THY or TSB medium. The following day, an overnight culture was diluted 1:15 in fresh medium and grown to A_{600} 0.3-0.4 (early exponential growth phase). The culture was centrifuged at 4000 x*g* for 10 min at RT and bacteria were then washed twice with PBS. The pellet was diluted in PBS to achieve 10% transmission at 600 nm, which corresponds to approximately 5x10^8 bacteria/ml. The bacterial suspension was further diluted in 1:100 ratio in EGM 2 supplemented with 2% fetal calf serum (FCS). 250 µl of this cell suspension was added to each well containing a semi-confluent monolayer of HUVEC, giving a multiplicity of

infection (MOI) 16:1. At 30 min post-infection, HUVEC were washed to remove extracellular bacteria and incubated for additional 30 min and 90 min. Following incubation, the cells were washed three times in EGM 2 basal medium and fixed either in 4% paraformaldehyde for fluorescence microscopy or in a fixative containing 5% formaldehyde and 2% glutaraldyhde in cacodylate buffer for electron microscopy. The cells were immuno-stained for immunofluorescence microscopic analysis as described in section 2.24. Images were captured using Zeiss AxioCam HRc 14-bit color CCD camera and Zeiss AxioVision 4.7 software and processed for contrast and brightness by GIMP 2.6.1

2.22.3 Interaction of recombinant protein coated latex beads with HUVEC

HUVEC were cultivated in EGM 2 supplemented with FCS, growth factors and penicillin/streptomycin (2.22.1). The cells were seeded into 24 well or 4 well plates containing sterile glass coverslips as described in section 2.22.2. The cells were grown to a semi-confluent monolayer on 12 mm diameter coverslips. For coating the latex beads with recombinant protein, approximately 10^7 polystyrene latex beads (3 μm and 1 μm diameter; Sigma) were washed three times in PBS and then coated overnight with 100 μg/ml of the recombinant full-length ScpC (EC-ScpC) and fusion protein constructs encompassing several domains of ScpC (EC-PR+A, and E-PR, EC-A+B/H) in PBS at 4°C. Affinity-purified recombinant GST was used for coating beads and served as a negative control. The recombinant protein coated beads were washed with PBS by centrifugation at 2500 rpm for 5 min. Beads were then washed twice in PBS and once in EGM 2 supplemented with 2% FCS as described above and the pellet was resuspended in 2-4 ml of EGM 2 supplemented with 2% FCS. 250 μl of the bead suspension was co-incubated with cells for 30 min, 1 h, 2 h and 3 h at 37°C under humidified environment containing 5% CO_2. After the incubation period, cells were washed several times with EGM 2 basal medium to remove unbound beads, fixed either in 4% paraformaldehyde dissolved in PBS (USB) and analysed under the light microscope.

For transmission electron microscopy, HUVEC were cultivated in a 6 cm cell culture dish, 1 ml of protein coated latex beads (3 μm; approx. 10^8 beads) were co-incubated with the cells for 2 h at 37°C under humidifiedenvironment containing 5% CO_2 and subsequently washed three times with EGM 2 basal medium. The cells were then fixed with 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer for electron microscopic analysis. For inhibition assay, the ScpC coated beads were pre-incubated with EGM 2 supplemented with 10% rabbit anti-ScpC IgG for 1 h at room temperature.

The coupling efficiency of recombinant protein with latex beads was checked by washing protein coated beads with PBS and then mixing in 1:1 ratio with SDS-PAGE loading buffer. Samples were boiled for 5 min at 95^{o}C and separated via SDS-PAGE. The activity of rScpC adsorbed to the surface of latex beads was checked by monitoring the IL-8 degradation by rScpC pre-coated on latex beads using human IL-8 Quantikine ELISA kit according to manufacturer's instructions. To study the uptake of recombinant protein without prior coating on latex beads, rScpC at concentration of 1-100 μg/ml was incubated with semi-confluent monolayer of HUVEC for a predetermined time period (1 h, 2 h and 3 h), cells were washed and then fixed in 4% paraformaldehyde. Cell bound ScpC coated latex beads and rScpC were visualized using rabbit polyclonal anti-ScpC IgG and anti-rabbit Alexa fluoro® 488 conjugated IgG and analysed under fluorescence microscope as describedin section 2.24.

2.22.4 Transmission electron microscopy

Cells were fixed in 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer and washed once with cacodylate buffer. Cells were then osmificated with 1% aqueous osmium for 1 h at room temperature and washed again. The pellets were embedded in 2% water agar and cut into small cubes. Samples were dehydrated with a graded series of acetone (10%, 20%, 50%) for 30 min on ice, followed by contrasting with 2% uranyl acetate in 70% acetone for overnight at 4° C. Samples were then further dehydrated with 90% and 100% acetone. Samples were infiltrated with the epoxy resin by applying 1 part 100% acetone and 1 part epoxy resin and incubated overnight, then 1 part 100% acetone and 2 parts epoxy resin for 8 h (Spurr, 1969). Pure resin was then added next day and incubated overnight. The samples were then placed into resin filled gelatine capsules and polymerized for 8 h at 75°C. Ultrathin sections were cut with a diamond knife, affixed to formvar-coated copper grids (300 mesh) and counterstained with 2% aqeuous uranyl acetate and lead citrate. After air-drying, samples were examined in a Zeiss transmission electron microscope (TEM910) at 80 kV acceleration voltage. The electron microscopic analysis and the preparation of the samples were kindly performed by Dr. Manfred Rohde (Helmholtz Centre for Infection Research, Braunschweig).

2.22.5 Antibiotic protection assay for the quantification of adherent and intracellular *S. pyogenes*

The number of viable intracellular streptococci were determined by an antibiotic protection assay as previously described by Valentin-Weigand et al. (1996). HUVEC were grown to confluency in 24 well cell culture plates as described in section 2.22.2. For the infection

assay, cells were washed once with EGM 2 basal medium, and 500 µl of EGM 2 supplemented with 2% fetal calf serum was added into each well. *S. pyogenes* strains were grown overnight in TSB at 37°C in a 5% CO_2 incubator. The overnight culture was centrifuged at 4000 rpm for 8 min and the inoculum was prepared as described in 2.22.2.1.

The bacteria were diluted in a 1:100 ratio in the EGM 2 supplemented with 2% FCS to give approximately $5x10^6$ bacteria/ ml. To calculate the exact colonies forming units (cfu) of the initial inoculum, serial dilutions of the inoculum were plated on blood agar plates. The confluent HUVEC monolayer was co-incubated with 250 µl of bacterial suspension for 2 h at 37°C under humidified conditions containing 5% CO_2. HUVEC were then washed twice with EGM 2 basal medium. Then, 500 µl of EGM 2 + 2 % FCS containing 100 µg/ml gentamicin and 5 µg/ml penicillin was added to each well and incubated for an additional 2 h at 37°C in a 5% CO_2 incubator to kill the extracellular bacteria. The cells were washed three times with EGM 2 basal medium and then 100 µl trypsin-EDTA solution was added into each well. After 5 min, 400 µl of 0.025% Triton X-100 (v/v) in PBS was added and serial dilutions of the cell lysate were plated in triplicate on blood agar plates and incubated for 24 h at 37°C. The next day, colony forming units were counted and intracellular bacteria were calculated compared to initial inoculum added into each well and expressed as percent invasion of bacteria into HUVEC.

The total number of adherent and invasive bacteria was determined by infecting the HUVEC monolayers with same amount of inoculum as described for the infection assay. The cells were lysed at 2 h post-infection in a same way as described above without pre-treatment of the cells with antibiotics. Accordingly, the serial dilutions of cell lysate were plated on blood agar plates in triplicate and incubated at 37°C for 24 h and colonies were counted the next day. The adherent bacteria were calculated by subtracting the number of invasive bacteria from the total number of cfu. The results were expressed as percent adherence of bacteria. Experiments were performed in triplicate and repeated three times.

2.23 *In vitro* assay with A549 lung epithelial cells

2.23.1 Cultivation of A549 lung epithelial cells

The A549 human lung epithelial cells were cultivated in DMEM supplemented with 10% FCS, 5 mM glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in humidified environment under 5% CO_2 conditions. The cells were cultivated to confluency and sub-cultured at an interval of 2-3 days. For sub-culturing, cells were washed once with

pre-warmed DMEM, incubated with 0.25% trypsin 1 mM Na-EDTA (trypsin-EDTA; PAN Biotech GmbH) to detach cells from the surface of the cell culture plate and 1 ml of cell suspension was then added to a new cell culture plate containing 15 ml of DMEM supplemented with antibiotics. Cells were cultivated at 37°C under humidified environment containing 5% CO_2 for 2-3 days. Cells were sub-cultured at an interval of 2-3 days. For latex beads assay, the cells were counted using a hemocytometer, diluted in antibiotic free DMEM supplemented with 10% FCS and glutamine (approximately 1.5 x 10^5 cells /ml) and seeded into a 24 well cell culture plate to give a semi-confluent layer of epithelial cells after 24 h.

2.23.2 Latex beads assay under acidic pH environmental conditions

The A549 lung epithelial cells were cultivated and seeded into 24 well cell culture plate as described above in section 2.23. On the next day, the cells were washed twice with DMEM supplemented with 1% FCS and 25 mM HEPES and incubated at 37°C under humidified environment containing 5% CO_2. Latex polystyrene beads were coated with rScpC as described above in section 2.22.3. To establish low pH environment conditions during the interaction of ScpC coated beads with host cells, the ScpC coated beads were centrifuged and the pellet was resuspended in DMEM + 1 % FCS diluted in 1:1 ratio in citric phosphate buffer (pH 5.5) to give final pH of 6.3. Subsequently, 250 µl of suspension was added to each well and incubated for 2 h at 37°C in 5% CO_2 incubator. After the incubation period, cells were washed 3-4 times with PBS to remove unbound beads and fixed in 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer, processed and analysed by scanning electron microscopy as described in section 2.17.3.

2.24 Double-immunofluorescence staining and fluorescence microscopy

2.24.1 Immunostaining to determine intracellular and extracellular bacteria

To distinguish intracellular bacteria from extracellularly located bacteria, infected cells were differentially stained with fluorophore-conjugated antibodies. The infected cells stored in 4% paraformaldehyde solution were washed twice with 500 µl of PBS. Non-specific binding sites were blocked by incubating the cells with 200 µl PBS supplemented with 10 % FCS for 1 h at room temperature (RT). Cells were incubated with rabbit anti-*S. pyogenes* polyclonal antibody (1:100 in PBS + 10% FCS) for 45 min at RT and washed two times with PBS. Then,

the secondary antibody, goat anti-rabbit Alexa fluor® 488 conjugated IgG (diluted 1:200 in PBS supplemented with 10 % FCS) was added and incubated for 45 min at RT. After the incubation period, cells were washed twice with PBS, permeabilized by addition of 0.1 % Triton X-100 in PBS for 5 min and washed two times with PBS. Intracellularly located bacteria were labeled by incubating the cells with the anti-*S. pyogenes* antibody for 45 min at RT, followed by incubation with goat anti-rabbit Alexa fluor® 568 conjugated IgG (diluted 1:300 in PBS + 10% FCS) for 45 min at RT. Hoechst (Molecular probes) was used to stain the nuclei of cells. Cells were incubated for 10 min with Hoechst (Molecular probes) diluted 1:5000 in PBS + 10% FCS and washed three times with PBS. The coverslips were mounted onto glass slides using prolong-gold antifade or Mowiol supplemented with N-propyl-gallate diluted in a ratio of 2:1 and sealed with nail polish. The samples were analysed using a fluorescence microscope (Zeiss Axiophot) as describedin 2.24.3.

2.24.2 Immunostaining for late endosomal/lysosomal compartments

To test the presence of *S. pyogenes* in a late endosomal/lysosomal compartment, monoclonal antibodies against human lysosomal-associated membrane protein 1 (LAMP-1) were used for staining. Following infection, cells were fixed in 4% paraformaldehyde diluted in PBS. The cells were washed twice with PBS and blocked with 10% FCS in PBS for 1 h at room temperature. After the incubation period, cells were washed twice with PBS, permeabilized by addition of 0.1 % Triton X-100 in PBS for 5 min and washed two times with PBS. The cells then incubated with rabbit anti-*S. pyogenes* antiserum/anti-ScpC IgG, diluted in 1:100 ratio in blocking buffer, for 45 min at room temperature. The secondary antibody, anti-rabbit Alexa fluor® 488 conjugated antibody were used to label *S. pyogenes* or rScpC coated latex beads. Then, LAMP-1 positive compartments were visualized by incubating the cells with mouse monoclonal anti-LAMP-1 antibodies (diluted 1:50 in blocking buffer) for 1 h at room temperature in a humid chamber. The cells were then incubated with anti-mouse Alexa fluor® 568 conjugated antibody for 45 min at RT. The coverslips were mounted on glass slides as described above in section 2.24.1 and examined by fluorescence microscopy.

Mowiol

20 g Mowiol
80 ml PBS, pH 7.3
40 ml glycerol
Centrifuged for 1 h at 20000 × g

Added 0.01M Sodiumazide to supernatant

N-propyl-gallate

0.1 M N-Propyl-Gallate

Dissolved in glycerol: PBS (1:9)

2.24.3 Fluorescence microscopy

For fluorescence microscopic analysis, the sample of interest was labeled with a fluorophore-conjugated antibody. The basic principle of a fluorescence microscope involves illuminating the sample with the light of specific wavelength, which is absorbed by the fluorophores and causes the sample to emit a longer wavelength light which is in the visible range of light spectrum. The components of a fluorescence microscope used include the mercury-vapor lamp as light source, the excitation filter**,** the dichroic mirror**,** and the emission filter. The filters and the dichroic mirror are specifically chosen to match the spectral excitation and emission characteristics of the fluorophore as described below in table 2. The epifluorescence microscope (Zeiss Axiophot) was used in this study. In epifluorescence microscopy, the light of a specific wavelength is passed through the objective and then onto the specimen instead of passing it first through the specimen. The fluorescence in the specimen gives rise to emitted light which is focused to the detector by the same objective that is used for the excitation. A filter between the objective and the detector filters out the excitation light from fluorescent light and only reflected excitatory light reaches the objective together with the emitted light. This method therefore gives an improved signal to noise ratio. The images were captured using a Zeiss AxioCam HRc 14-bit color CCD camera and Zeiss AxioVision 4.7 software.

Table 2

Filter	**Excitation wavelength**	**Emission wavelength**	**Fluorochrome**
Nr. 01	365 nm	397 nm	Hoechst
Nr. 10	450-490 nm	515-565 nm	Alexa Fluor® 488
Nr. 15	546-512 nm	590 nm	Alexa Fluor® 568

2.25 Statistical Analysis

The data were compared by unpaired Student's t-test using GraphPad Prism 5 and the P-value <0.05 was considered significant.

3 Results

3.1 Recombinant expression and characterization of ScpC

3.1.1 ScpC cleaves chemoattractant interleukin-8

In order to investigate whether ScpC *per se* is responsible for the IL-8 degrading protease activity displayed by M3 serotype *S. pyogenes*, a Δ*scpC* single gene mutant of M3 serotype *S. pyogenes* invasive strain A475 was analysed for its ability to degrade IL-8 in comparison to wildtype (WT) A475 *in vitro*. For this, a cytokine ELISA was performed for quantitative determination of IL-8 in the culture supernatant of WT A475 and its isogenic Δ*scpC* mutant after co-incubating the supernatant with recombinant IL-8 (100 ng/ml). Our data showed a significant ($P<0.001$) reduction in the amount of IL-8 after co-incubation with the culture supernatant of WT A475 in comparison to IL-8 co-incubated with growth medium alone, indicating that IL-8 is degraded by the A475 culture supernatant. However, in contrast, when recombinant IL-8 was co-incubated with the supernatant of its isogenic Δ*scpC* mutant strain, there was no reduction in the amount of IL-8, confirming that the genetic deletion of *scpC* gene results in the elimination of IL-8 proteolytic activity (Figure 7A). The *in vitro* degradation of IL-8 by *S. pyogenes* A475 and the complete lack of IL-8 degrading ability of its isogenic Δ*scpC* mutant were further shown by SDS-PAGE analysis (Figure 7B). Thus, by using a *scpC* single gene deletion mutant of M3 serotype *S. pyogenes* invasive isolate A475, this study showed that ScpC is responsible for the IL-8 proteolytic activity of M3 *S. pyogenes*. This finding is in accordance with the previous reports on the role of ScpC in IL-8 degradation by M14 and M1 serotype *S. pyogenes* (Hidalgo-Grass et al., 2006; Zinkernagel et al., 2008).

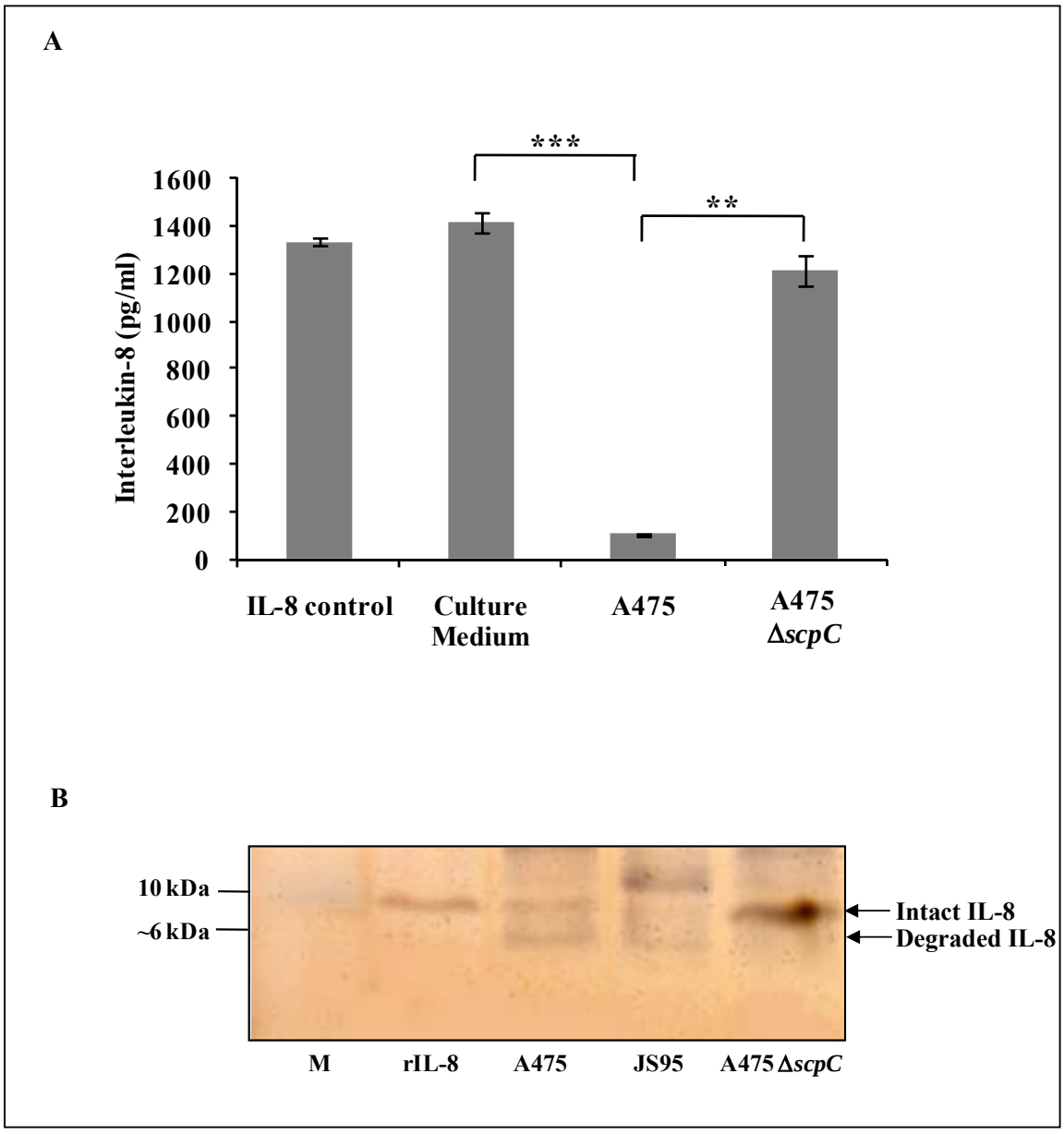


Figure 7. IL-8 degrading ability of wildtype M3 serotype *S. pyogenes* strain A475 and its isogenic Δ*scpC* mutant. A) The degradation of IL-8 by M3 serotype *S. pyogenes* strain A475 and the lack of IL-8 proteolytic activity of its isogenic Δ*scpC* mutant is shown by cytokine ELISA. The data represent mean ± standard deviation of three independent experiments. Error bars represent standard deviation. The data were compared by utilizing an unpaired Student's t-test and a P-value <0.05 was considered significant. B) Silver stained SDS-PAGE gel showing the cleavage of IL-8 into a smaller peptide fragment after co-incubation with the M3 *S. pyogenes* supernatant. The A475 Δ*scpC* mutant strain lacks the IL-8 proteolytic activity as shown by the presence of a band of approximately 8 kDa, corresponding to intact IL-8. High level ScpC expressing M14 *S. pyogenes* strain JS95 was taken as a positive control.

3.1.2 Recombinant expression and purification of biologically active ScpC

3.1.2.1 Cloning, heterologous expression and purification of recombinant ScpC

S. pyogenes strains isolated from patients with invasive disease were anlysed for the presence of the *scpC* gene via polymerase chain reaction (PCR). The *scpC* gene was found to be present among all *S. pyogenes* strains tested (Figure 8). Multiple sequence alignment of the *scpC* from all available *S. pyogenes* genomes indicated >98% sequence similarity. Previous studies have shown IL-8 degrading activity of partially purified ScpC (SpyCEP) from the culture supernatant of *S. pyogenes* strain H292 (Edwards et al., 2005). To further characterize the ScpC protease and to study the role of ScpC in *S. pyogenes* pathogenesis of invasive diseases, we recombinantly expressed ScpC in *Escherichia coli (E. coli*). The *scpC* open reading frame encodes a 1458 amino acid protein with an approximate molecular mass of 150 kDa. For recombinant expression of ScpC, the full-length *scpC* excluding regions encoding pre-pro domain and cell wall-anchoring domain was amplified from the *S. pyogenes* invasive isolate A416 by PCR using primers which incorporated the appropriate restriction sites at 5' and 3' end of the full-length *scpC* (see section 2.13.1). The sequence of the *scpC* gene in the vector was verified by DNA sequencing.

The *scpC* was heterologously expressed in *E. coli* strain M15 containing plasmid pREP4 using a pQE-60 expression vector. pQE-60 expresses the recombinant ScpC with a 6xHistidine (6xHis) affinity tag placed at the C-terminus of the protein. In pQE-60, expression of recombinant protein is induced by the addition of an analogue of lactose, isopropyl-β-D-thiogalactose (IPTG). Therefore, the *E. coli* cells transformed with pQE-60 derivative containing *scpC* were induced with IPTG for 4 h. The recombinantly expressed ScpC was purified from the bacterial cell lysate by using immobilized metal affinity chromatography and the protein was eluted under native conditions as described in section 2.13.2. Recombinant ScpC tagged with 6xHis was purified from the bacterial lysate via binding to Nickel-nitrilotriacetic acid (Ni-NTA) agarose (see section 2.13.2). The western immunoblotting using horseradish peroxidase-conjugated Ni-NTA, which is used for the direct detection of 6xHis tagged protein, recognized the band with a molecular weight of approximately 150 kDa in the cell lysate of *E. coli* transformants (Figure 9A), indicating the expression of full-length 6xHis tagged ScpC. The size of the purified recombinant ScpC protein, as estimated by SDS-PAGE, was 120-150 kDa, which is close to its expected molecular mass (Figure 9B).

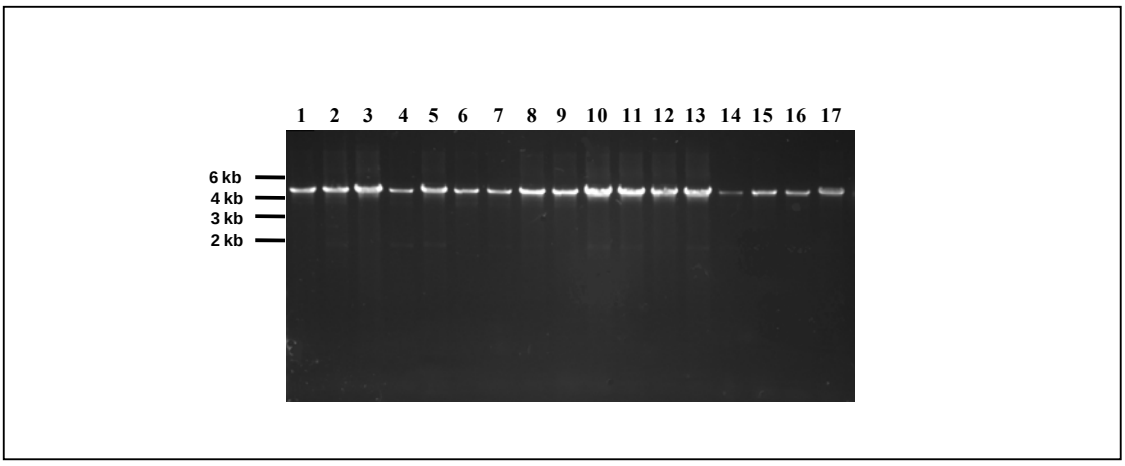


Figure 8. **PCR analysis for screening of the *scpC* gene in different M serotype *S. pyogenes* strains.**

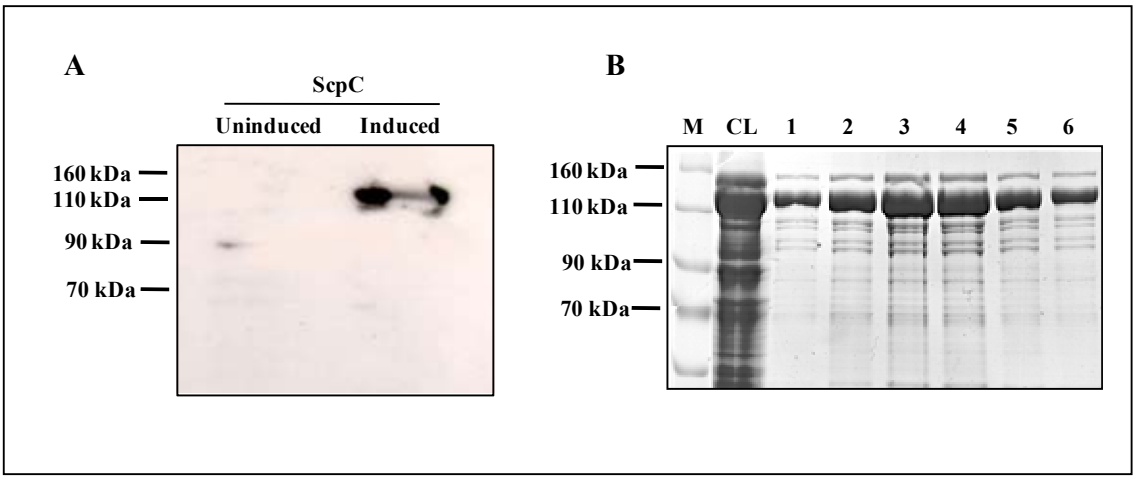


Figure 9. Cloning and recombinant expression of ScpC A) *E. coli* cells transformed with pQE60 derivative harboring the *scpC* gene were incubated with IPTG for 4 h. *E. coli* cells collected before and after induction with IPTG were lysed and separated on SDS-PAGE gel. Protein was transferred to a nitrocellulose membrane and detected using Ni-NTA conjugate. B) SDS-PAGE analysis of the *E. coli* cell lysate showing the expression of recombinant protein with molecular weight of approximately 120-150 kDa. Lane 1-6 refers to the fractions collected after purification of recombinant protein from bacterial crude extracts using Ni-NTA agarose.

3.1.2.2 NH_2-terminal amino acid sequencing of recombinant protein

Edman degradation sequencing procedure was followed for determining the N-terminal amino acid sequence of purified recombinant ScpC. When separated on SDS-PAGE gel, the recombinant protein showed two bands which corresponded to molecular weights of approximately 150 kDa and 120 kDa (Figure 9B). The protein was then electrotransferred

onto PVDF membrane for 2 h and stained. Both, the upper and lower band was excised individually from the membrane and sequenced. Followed by the Edman degradation chemistry, amino acids were analysed by high performanceliquid chromatography (HPLC). The first ten NH_2-terminal amino acid residues of 120 kDa band were identified as indicated below. The sequence alignment of amino acids residues deduced by Edman degradation with the ScpC peptide sequence from NCBI protein data bank (accession number ABA33824.1) corresponded to the 245-255 amino acid residues of ScpC (Figure 10). The amino acid sequence of 150 kDa band could not be determined due to low protein concentration.

```
          MRISDVSTAKVKSKEDMLARQKAAGINYGSWINDKVVFAH    200
          ....................................

          NYVENSDNIKENQFEDFDEDWENFEFDAEAEPKAIKKHKI    240
          ....................................

ScpC      YRPQSTQAPKETVIKTEETDGSHDIDWTQTDDDTKYESHG    280

rScpC     ....STQAPKETVI.........................
```

Figure 10. **Amino terminal amino acid sequence of recombinant ScpC**. The multiple sequence alignment of the first ten amino acid residues of rScpC identified by edman degradation with the peptide sequence of ScpC of *S. pyogenes* strain JS95.

3.1.2.3 Proteolytic activity of purified recombinant ScpC

The proteolytic activity of purified recombinant ScpC was examined by monitoring its IL-8 degrading ability. To determine the IL-8 proteolytic activity of purified rScpC, rScpC and its serial dilutions were incubated with recombinant IL-8 for 16 h at 37^{o}C and residual IL-8 was measured with cytokine ELISA. Incubation of purified rScpC with IL-8 showed significant reduction in the amount of IL-8 in comparison to IL-8 incubated with PBS, indicating that rScpC is functionally active. Incubation of 3.0 µg of rScpC with recombinant IL-8 (100 ng/ml) cleaved almost 99% of IL-8. IL-8 proteolytic activity could also be detected at lower recombinant protein concentration, with reduction in the amount of rScpC to 0.003 µg showed efficient degradation of IL-8 (Figure 11A). Western immuno-blotting further confirmed the retained IL-8 proteolytic activity of rScpC as shown by the loss of the 8 kDa band corresponding to IL-8 after co-incubation with purified rScpC as well as with the culture supernatant of invasive M14 serotype *S. pyogenes* strain JS95. In contrast, co-incubation of IL-8 with supernatant of *scpC* mutant of JS95 (JS95 Δ*scpC*/Δ*scpA*; kindly provided by Prof.

Emanuel Hanski, The Hebrew University-Hadassah Medical School, Jerusalem, Israel) retained a band of approximately 8 kDa, corresponding to intact IL-8 (Figure 11B).

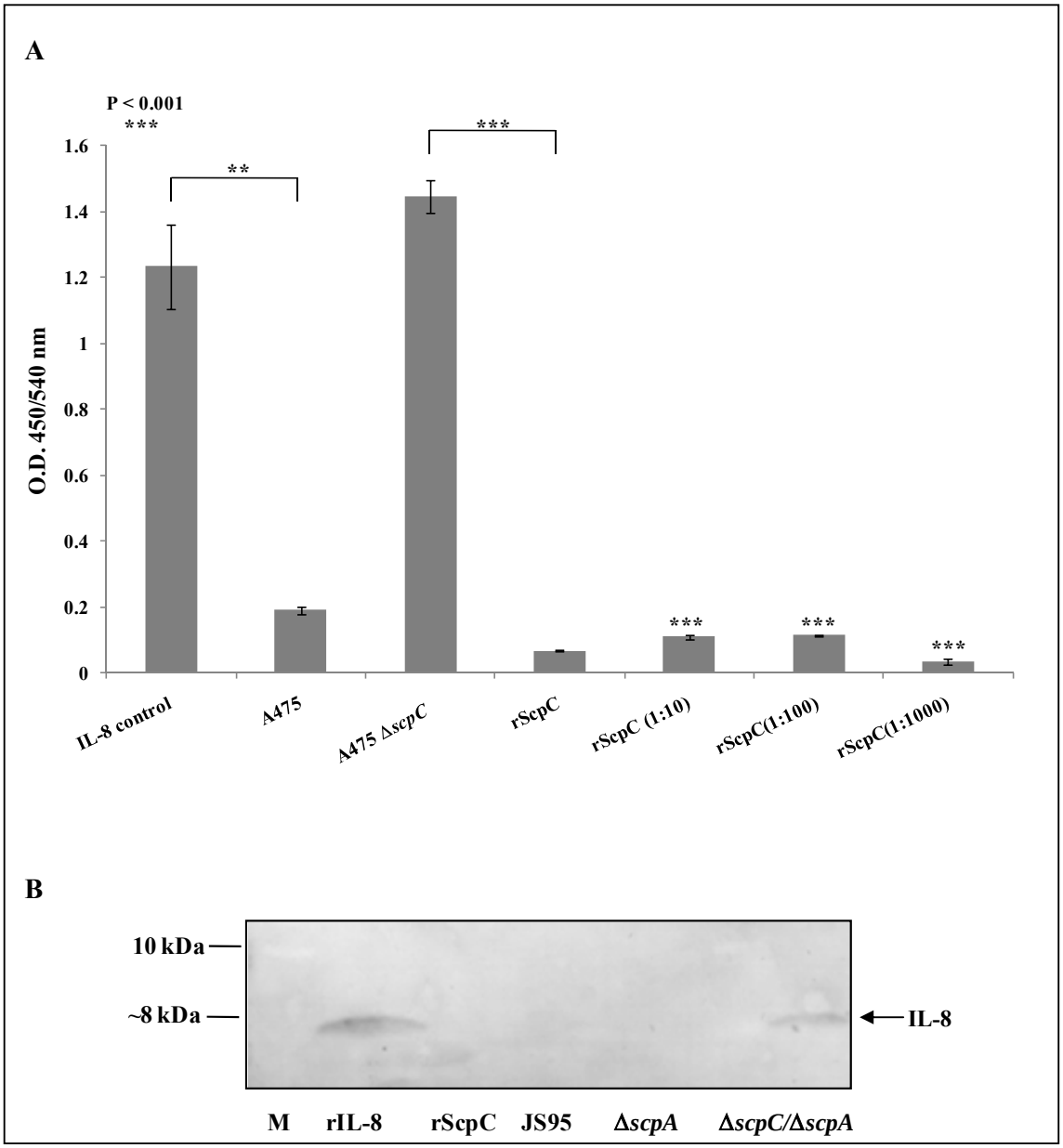


Figure 11. Cleavage of IL-8 by recombinant ScpC. A) The purified rScpC and its diluted fractions significantly degraded IL-8 when co-incubated with recombinant IL-8 for 16 h at 37°C in comparison to A475 Δ*scpC* mutant, which lacks the IL-8 proteolytic activity, indicating that rScpC is enzymatically active. The culture supernatant of WT A475 efficiently degraded IL-8, which served as a positive control. B) The 8 kDa band corresponding to IL-8 disappeared when co-incubated with rScpC or with WT *S. pyogenes* JS95 for 16 h, due to the degradation of IL-8. The JS95 Δ*scpC*/Δ*scpA* double mutant did not possess IL-8 proteolytic activity as the 8 kDa band was retained on western immunoblot. The JS95 Δ*scpA* mutant strain served as a control.

3.1.3 Raising polyclonal antibodies against ScpC and purification of specific IgG

The immunogenicity of ScpC in humans and mice was identified by Lei et al. (2000). Rodriguez-Ortega et al. (2006) have suggested a potential role of specific antibodies against a putative cell envelope protease Spy0416, a homologue of ScpC in M23 *S. pyogenes*, in host-pathogen interaction based on the observed protective effect of ScpC specific antibodies against *S. pyogenes* infections. Therefore, to investigate the contribution of ScpC specific antibodies in combating *S. pyogenes* infection, polyclonal antibodies against the purified recombinant ScpC protein were raised in a rabbit. The rabbit anti-ScpC serum showed immuno-reactivity with rScpC on the dot blot (Figure 12) as well as on western blot. Antiserum did not cross react with recombinant GST or *S. pyogenes* M1 protein, which was taken as negative control. Also, rabbit pre-immunization sera did not cross react with rScpC or *S. pyogenes* culture supernatant. The immunoglobulin Gs (IgG) were isolated from antiserum by affinity chromatography using protein A agarose. $F(ab')_2$ fragment of anti-ScpC IgG was generated by papain digestion and separated from Fc fragment. Purified IgG could detect as low as 0.001 μg of rScpC at dilutions ranging from 1:100 to 1:5000.

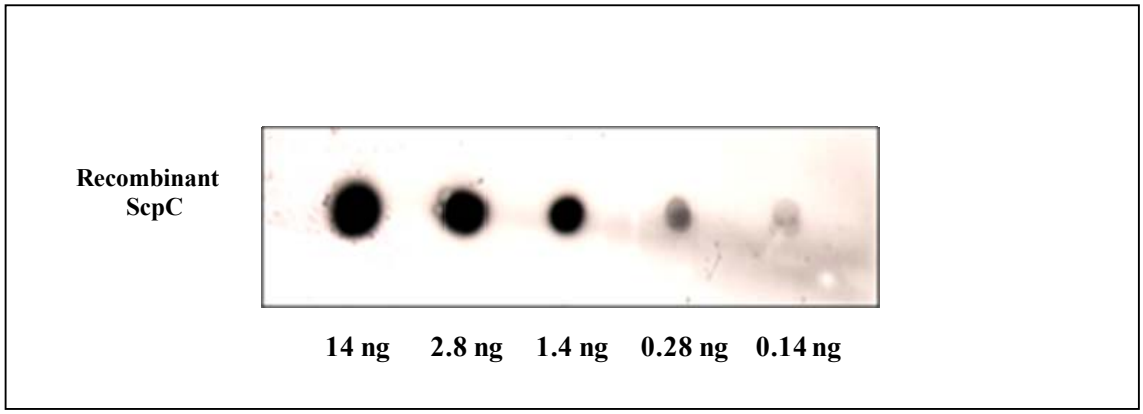


Figure 12. Recombinant ScpC is highly immunogenic in rabbit. The purified rScpC and its serial dilutions were incubated with antiserum containing specific antibodies against rScpC. Recombinant ScpC was recognized by anti-ScpC antiserum as shown by the dot-blot analysis.

3.1.4 Bacterial growth in liquid culture

The growth behavior of WT M3 serotype *S. pyogenes* strain A475 and its isogenic Δ*scpC* mutant strain was compared *in vitro*, to check if the inactivation of *scpC* gene has affected the metabolic growth rate of the mutant strain in comparison to parental strain. The WT A475 and

isogenic Δ*scpC* mutant showed equivalent logarithmic growth in Tryptic Soy Broth (TSB) when grown at 37°C in 5% CO_2 incubator. The generation time of WT parental strain A475 and isogenic Δ*scpC* mutant strain was about 45 min in exponential phase (Figure 13).

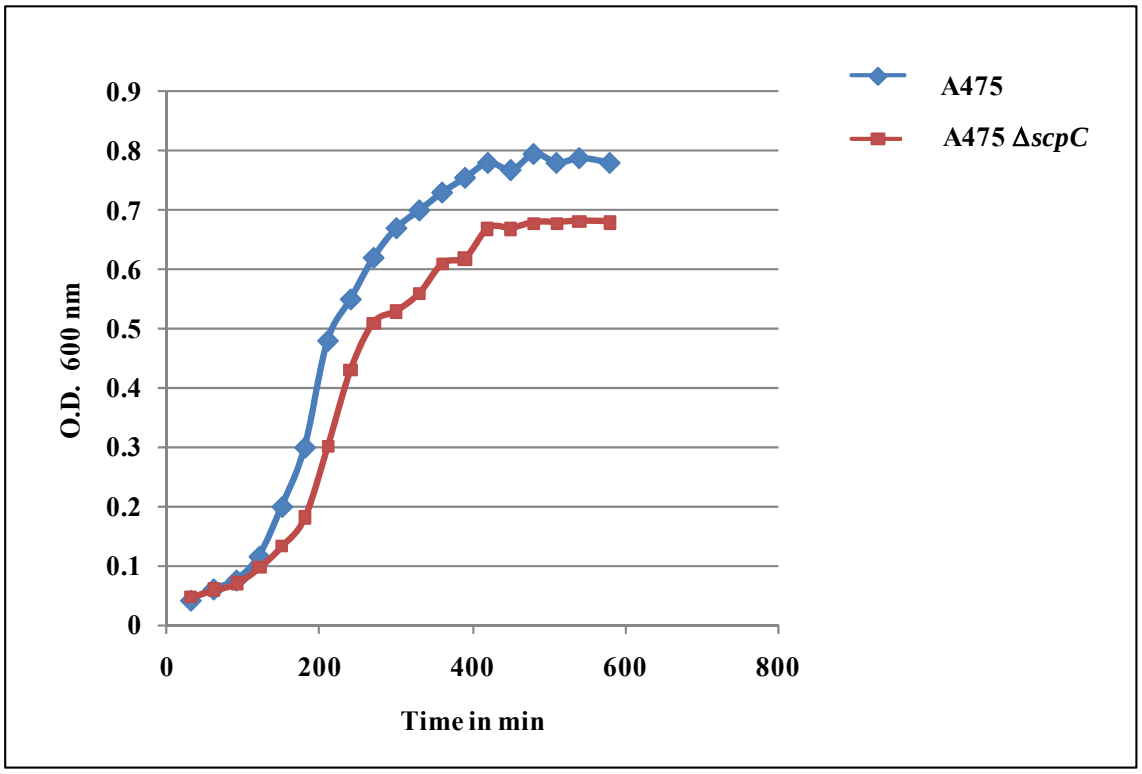


Figure 13. **Growth curve of *S. pyogenes* A475 and isogenic Δ*scpC* mutant strain**. Strains were grown in TSB medium and optical density was measured at 600 nm at 30 min time interval.

3.1.5 Localization and reassociation of ScpC on the *S. pyogenes* cell surface

3.1.5.1 Localization of ScpC on the cell surface of *S. pyogenes*

ScpC shows homology with various extracellular cell wall-anchored proteins of Gram-positive bacteria including C5a peptidase of *S. pyogenes*, CspA of *S. agalactiae*, cell envelope protease PrtP of non-pathogenic *Lactococcus lactis* and PrtS of *Streptococcus thermophilus* (Harris et al., 2003; Fernandez-Espla et al., 2000; Siezen et al., 1999). These surface-anchored proteins usually have a conserved C-terminal sorting signal with a LPXTG motif which is required for sortase-mediated cleavage and subsequent covalent cross linking of the protein on the cell surface. Based on the predicted LPXTG motif containing C-terminal sequence of ScpC, which is required for the cell wall-anchoring, we investigated whether ScpC is located on the cell surface of *S. pyogenes*. For this, IL-8 proteolytic activity was determined in the *S.*

pyogenes culture supernatant as well as in the pellet containing bacterial cells grown to mid-exponential phase. Among the *S. pyogenes* strains (M1 serotype KTL3, M3 serotype A475 and M14 serotype JS95) tested, IL-8 proteolytic activity was detected both as cell-associated (pellet fraction) and secreted form (supernatant fraction). Among these strains, M1 serotype *S. pyogenes* strain KTL3 showed significantly higher cell-associated IL-8 proteolytic activity in comparison to the presence of proteolytic activity in bacterial growth medium (Figure 14) indicating that a major fraction of ScpC is cell-associated.

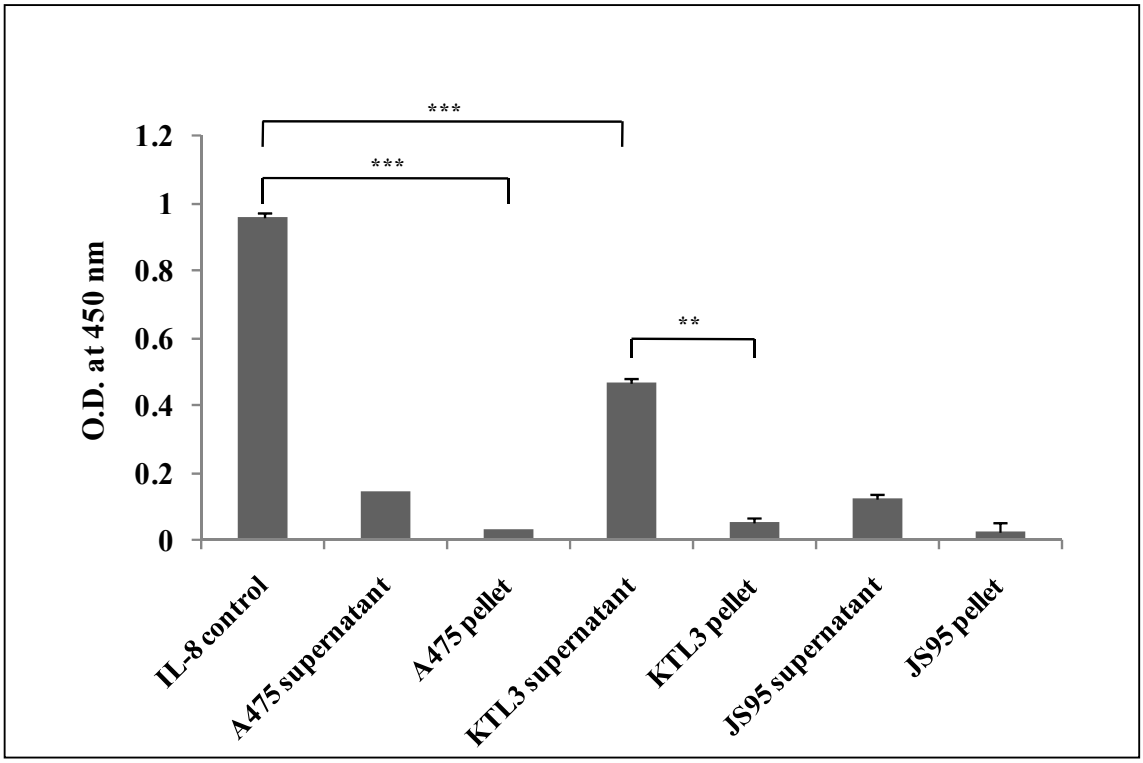


Figure 14. **ScpC protease is secreted as well as cell surface-anchored**. The culture supernatant and the bacterial cells of *S. pyogenes* strains M3 serotype A475, M1 serotype KTL3, and M14 serotype JS95 were collected after pelleting the exponential phase culture medium. The culture supernatant and bacterial cells were incubated with recombinant IL-8 for 16 h at 37^{o}C. To examine the presence of the IL-8 degrading proteolytic activity, the amount of IL-8 was determined by using a cytokine ELISA. The IL-8 proteolytic activity was detected in the culture supernatant as well being associated with *S. pyogenes* cells.

To examine the cell surface localization of ScpC, *S. pyogenes* strains of serotype M1, M3, M14 and M18 were immunolabeled with the affinity-purified rabbit polyclonal anti-ScpC IgG and anti-rabbit Alexa fluor® 488 conjugated secondary antibody and then analyzed by immunofluorescence microscopy. A strong fluorescence signal was observed co-localizing

with *S. pyogenes* incubated with anti-ScpC IgG, indicating that ScpC is a cell surface-anchored protein (Figure 15). Immunostaining of whole cells of WT *S. pyogenes* M14 JS95 and M3 A475 with rabbit polyclonal anti-ScpC IgG also revealed the presence of ScpC on the cell surface (Figure 18 A&D). In contrast, the respective isogenic Δ*scpC* mutant strains showed no fluorescence signal indicating that fluorescence emission by the WT *S. pyogenes* strains is due to the specific interaction of anti-ScpC antibody with the surface-bound ScpC (Figure 18 B&E).

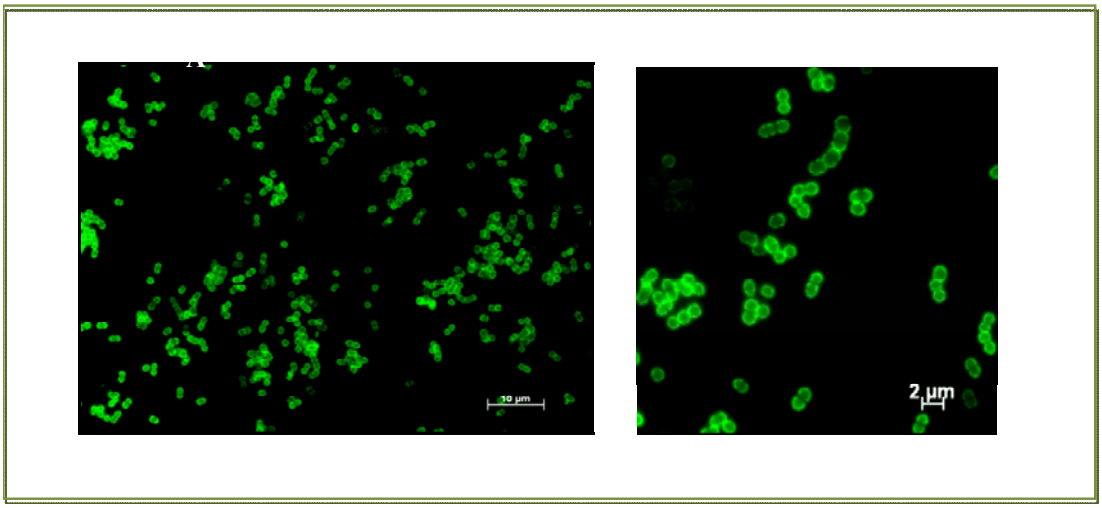


Figure 15. Localization of ScpC on the cell surface of M18 *S. pyogenes*. Whole cells of highly encapsulated M18 serotype *S. pyogenes* were immunolabeled with anti-ScpC IgG. The anti-rabbit Alexa fluor® 488 conjugated secondary antibody was used to detect the bound IgG.

Surface localization of ScpC was further confirmed by field emission scanning electron microscopy (FESEM). Whole cells of streptococci were immunolabeled with polyclonal anti-ScpC IgG. Bound IgG were visualized by incubating with protein A-coated gold particles and analysed by scanning electron microscopy. Confirming the results of immunofluorescence microscopic analysis, the gold particles were associated with the surface of M14 *S. pyogenes* strain JS95 (Figure 16A) as well as with the surface of M3 *S. pyogenes* strain A475 (Figure 16C), but not with the respective isogenic Δ*scpC* mutant strains (Figure 16 B&D). Together, these results strongly indicate that ScpC is localized on the cell surface of *S. pyogenes*.

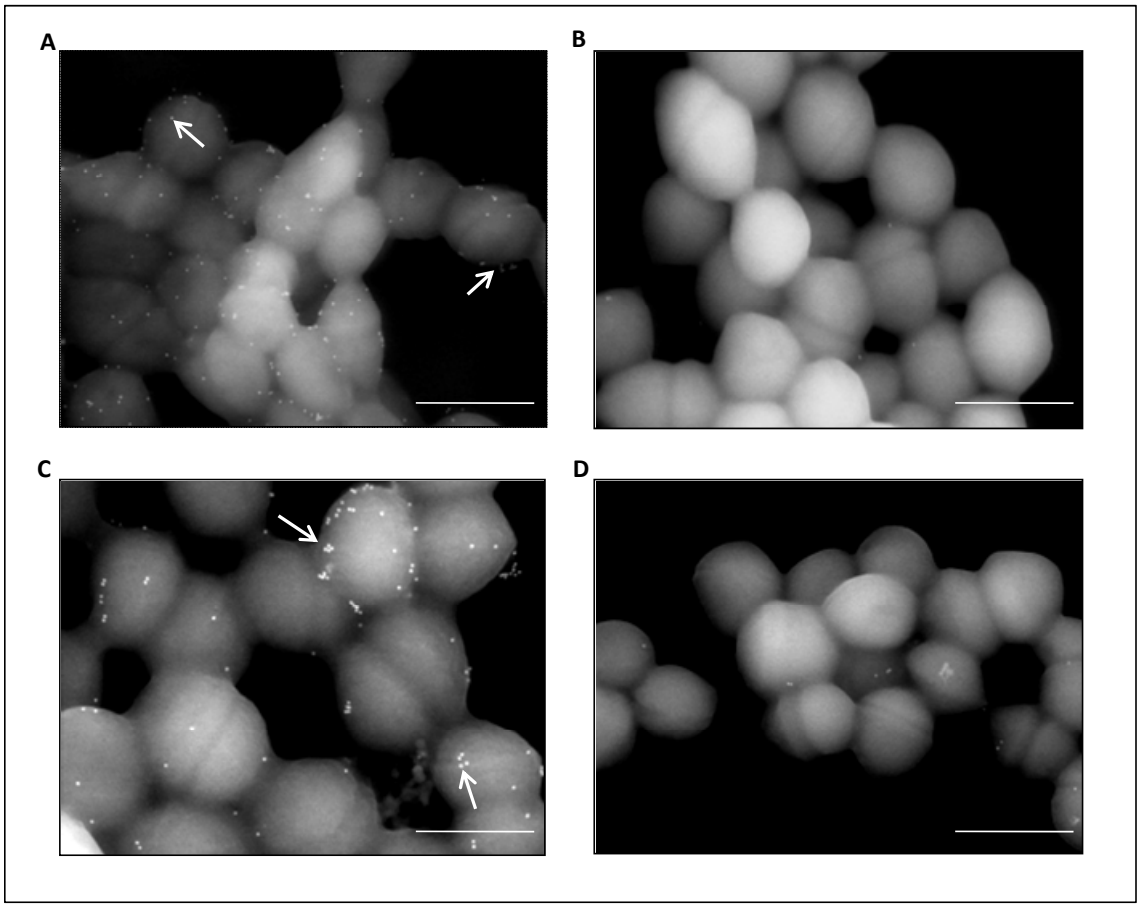


Figure 16. FESEM analysis of the localization of ScpC on the *S. pyogenes* cell surface. WT *S. pyogenes* JS95, A475 and the respective isogenic Δ*scpC* mutant strains were immuno-gold labeled with specific antibodies raised against ScpC and protein A-gold particles. FESEM depicts the presence of gold particles on the cell surface of JS95 and A475 indicating that ScpC is located on the cell surface (A&C). In contrast, gold particles did not associate with the surface of JS95 Δ*scpC*/Δ*scpA* and A475 Δ*scpC* mutant strains indicating the lack of surface expression of ScpC (B&D). Arrows indicates the association of gold particles to the surface of *S. pyogenes*. Bar corresponds to 0.5 μm (C) and 1 μm (A-B-D).

The observation that the fluorescence signal intensity emitted by *S. pyogenes* varies among the *S. pyogenes* serotypes tested (M1, M3, M14, M18) indicated the differential surface expression levels of ScpC by these strains. *S. pyogenes* strain JS95 emitted a stronger fluorescence signal in comparison to A475, indicating the higher level of surface expression of ScpC by JS95 (Figure 18 A&D). M18 serotype *S. pyogenes* A471, a high capsule producing strain emitted an intense fluorescence signal, pointing towards a high level expression of ScpC on the cell surface (Figure 15). In contrast, M1 *S. pyogenes* strain A270 showed much less surface expression level of ScpC (data now shown). To determine the surface expression of ScpC by JS95, a flow cytometer was used to collect 10,000 events for

each strain. A higher level of surface expression of ScpC by JS95 during the early exponential phase of the growth was observed, however very little fluorescence signal was detected in the late exponential phase or stationary phase (Figure 17). Previous studies have also described that the expression of ScpC is highly upregulated in *S. pyogenes* invasive isolates in comparison to pharyngeal isolates, indicating the differential regulation of ScpC expression (Sumby et al., 2006). Thus, the present data further confirmed that the expression of ScpC is highly regulated in *S. pyogenes* strains and may explain the difference in the ability of *S. pyogenes* strains to evade the immune response and to cause invasive diseases.

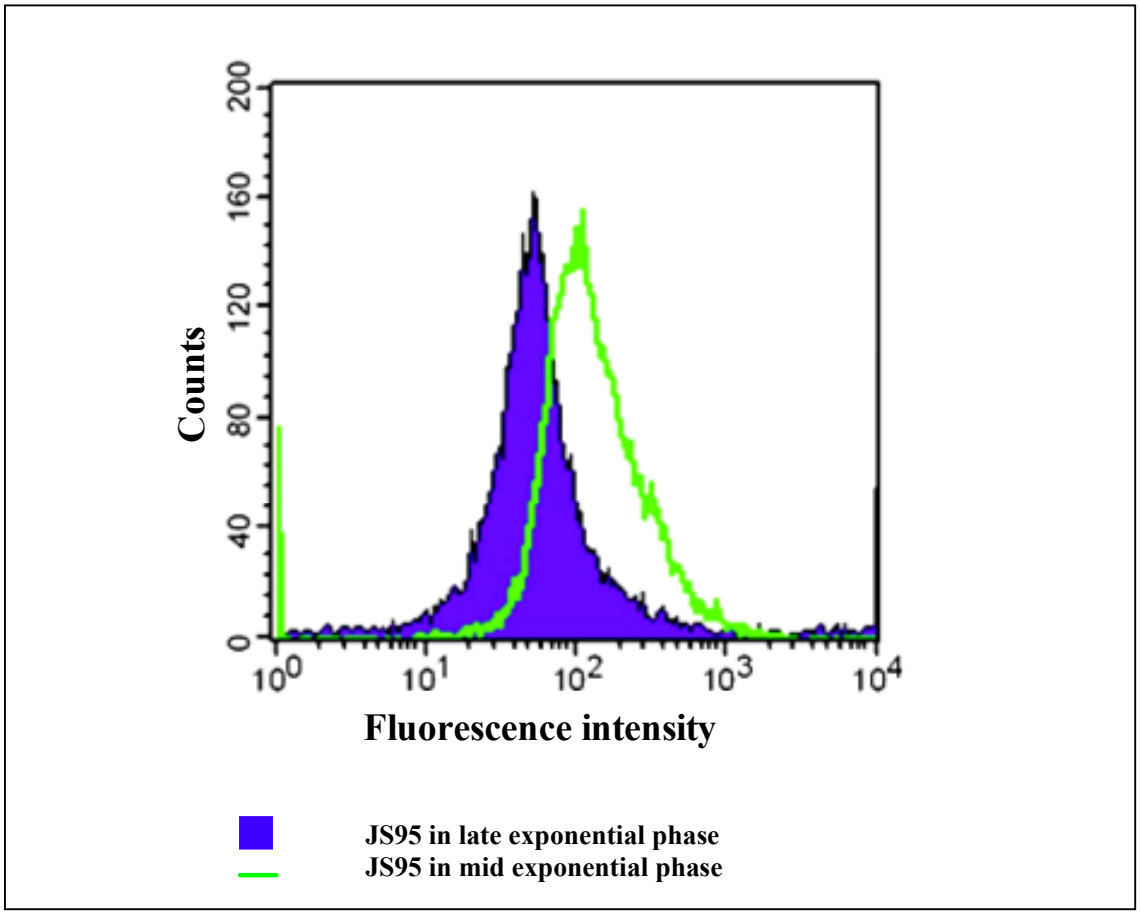


Figure 17. Surface expression of ScpC is growth phase dependent. *S. pyogenes* JS95 were grown to mid-exponential phase (A_{600nm} 0.4) and late-exponential phase (A_{600nm} 0.85). After washing the bacterial cells with PBS, cells were labeled with rabbit polyclonal anti-ScpC antibody and anti-rabbit Alexa fluor® 488 conjugated secondary antibody. Samples were analysed by using a flow cytometer. Data shows change in mean fluorescence intensity between JS95 in late exponential phase and JS95 in mid exponential phase.

3.1.5.2 Reassociation of ScpC with the *S. pyogenes* cell surface

Various cell surface-anchored glycolytic proteins including surface dehydrogenase (SDH) and surface enolase (SEN) have been shown to be essential for the overall virulence of *S. pyogenes* (Boel et al., 2005). These proteins interact with extracellular matrix proteins and described to be localized on the surface by reassociation (Chhatwal, 2002). On the basis of the observed cell surface localization of ScpC in previous results, we anticipated that ScpC might reassociate to the cell surface of *S. pyogenes*. As demonstrated before, ScpC proteolytic activity can also be detected in the culture supernatant of *S. pyogenes* (Figure 14), suggesting that ScpC is secreted into the extracellular environment. Therefore, we next tested whether secreted ScpC can subsequently be displayed on the cell surface of *S. pyogenes* via reassociation. To examine this, we incubated the JS95 Δ*scpC*/Δ*scpA* and A475 Δ*scpC* bacterial cells with the culture supernatant of WT JS95 grown to mid-exponential phase for 1 h. The WT JS95 showed high IL-8 proteolytic activity in the supernatant as well as high level of expression of ScpC on the cell surface in early to mid-exponential phase of growth (Figure 14). Interestingly, when respective isogenic Δ*scpC* mutant strains, pre-incubated with supernatant of the WT JS95, were subsequently immunolabeled with rabbit polyclonal anti-ScpC IgG and secondary fluorophore-conjugated antibody, a strong fluorescence signal was emitted by bacterial cells of Δ*scpC* mutant strains (Figure 18 C&F). The JS95 Δ*scpC* and A475 Δ*scpC* mutant strains, without prior incubation with the supernatant of WT JS95, did not emit any fluorescence signal when immunostained with anti-ScpC IgG and fluorophore-conjugated secondary antibody (Figure 18 B2&E2). As a control, WT parental strains and isogenic Δ*scpC* mutant strains incubated only with anti-rabbit fluorophore-conjugated antibody gave no background signal. This data indicated that ScpC could reassociate with the surface of mutant strains when these bacteria were incubated with the supernatant of WT strain as a natural source of ScpC.

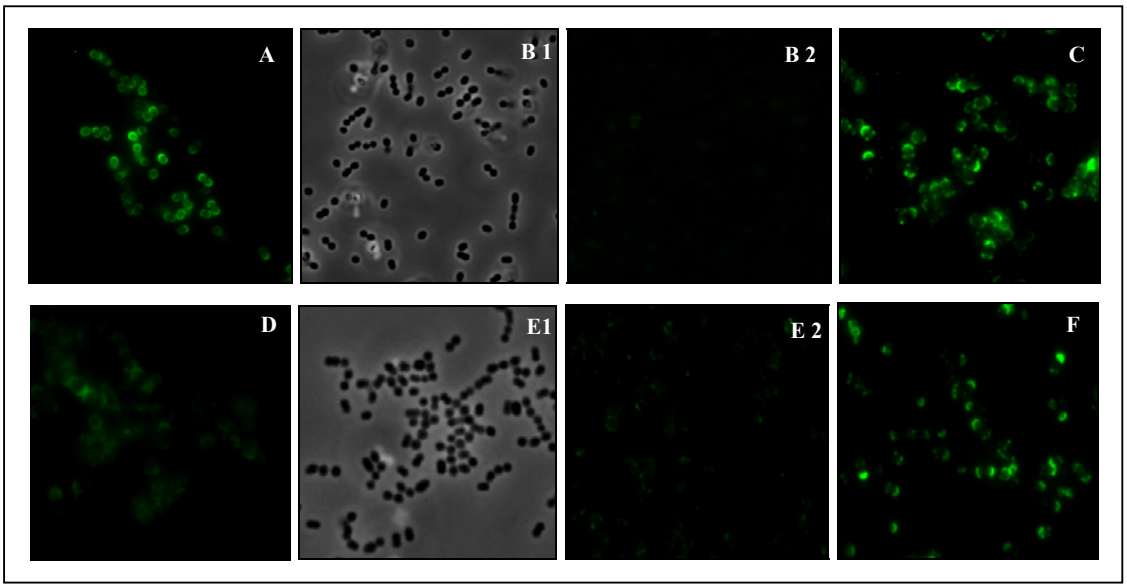


Figure 18. Surface localization and reassociation of ScpC. *S. pyogenes* strains were incubated with anti-ScpC IgG for 1 h at RT. The anti-rabbit Alexa fluor® 488 conjugated antibody was used to visualize the cell bound IgG. Surface expression of ScpC was indicated by the fluorescence signal emitted by WT JS95 and A475 strains (A&D). In contrast, no signal was shown by the respective isogenic Δ*scpC* mutant strains (B2&E2). The phase contrast images (B1&E1) of respective isogenic Δ*scpC* mutant indicate that despite the presence of bacteria, no fluorescence signal was emitted (B2&E2). For testing reassociation, the cells of JS95 Δ*scpC* and A475 Δ*scpC* mutant were incubated with the supernatant of WT JS95 growth medium for 2 h. The bacterial cells were washed twice in PBS, stained as described above and analysed under the fluorescence microscope. The mutant strains showed enhanced fluorescence signal intensity indicating the reassociation of secreted ScpC on the cell surface (C&F).

Consistently, immuno-FESEM revealed the reassociation of ScpC with the cell surface of JS95 Δ*scpC* (Figure 19C) and A475 Δ*scpC* mutant (Figure 19E) when incubated with the culture supernatant of ScpC expressing WT JS95. Very low or almost no surface expression of ScpC was observed on the JS95 Δ*scpC* mutant (Figure 19B) and A475 Δ*scpC* mutant (Figure 19D) incubated with growth medium alone, which served as control. The low background label of gold particles on cell surface of A475 Δ*scpC* mutant might not be due to the ScpC expression since this strain was unable to degrade IL-8 (see section 3.1.1, Figure 7). However, this mutant still produces a small N-terminal fragment of ScpC, which might explain its weak interaction with anti-ScpC IgG. These results indicate that ScpC can reassociate with the cell surface of *S. pyogenes* after being secreted into the extracellular

environment and suggests that ScpC is able to reassociate either with the surface of a producing strain via binding-back or with the surface of other bacteria present in the local area of infection.

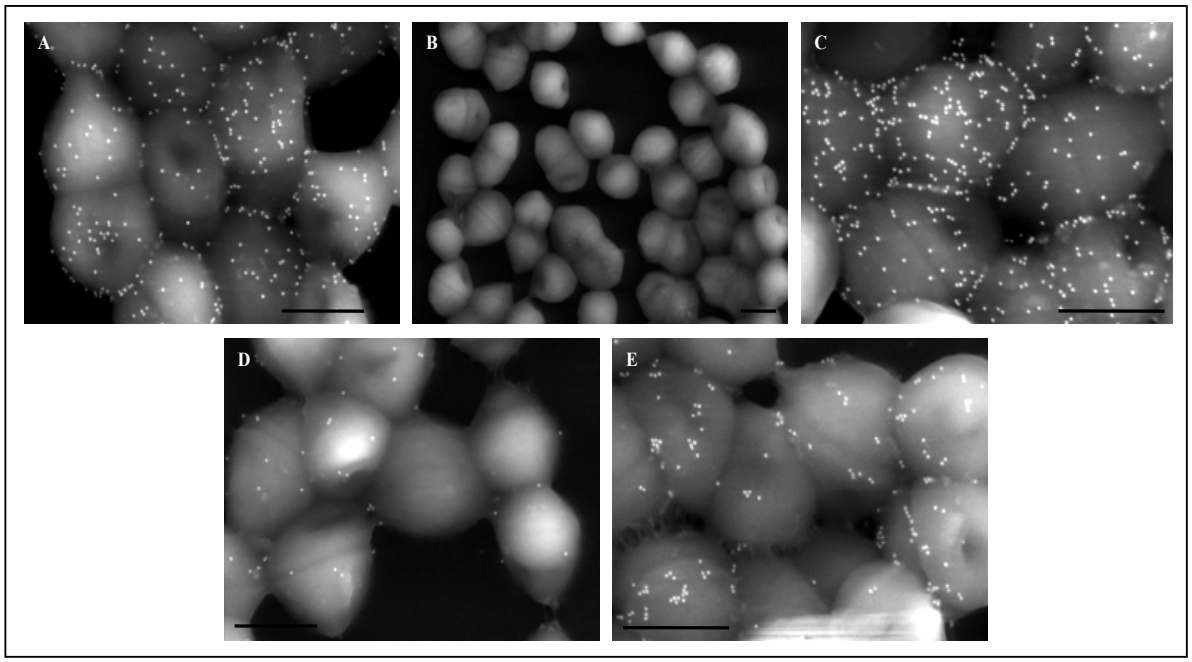


Figure 19. Reassociation of ScpC with the *S. pyogenes* cell surface: High level surface expression of ScpC was observed on WT JS95 (A). The bacterial cells of JS95 Δ*scpC* and A475 Δ*scpC* mutant strain were incubated with the supernatant of WT JS95 culture medium for 2 h and washed with PBS. The cells were immuno-gold labeled by using anti-ScpC antibody and protein A-coated gold particles. FESEM analysis shows reassociation of secreted ScpC with the cell surface of JS95 Δ*scpC* mutant strain and A475 Δ*scpC* mutant strain (C&E). The JS95 Δ*scpC* mutant did not show localization of gold particles on the surface (B) and very low background labeling in case of A475 Δ*scpC* mutant was observed (D). Each bar corresponds to 0.5 μm.

3.1.6 Cloning, overexpression and purification of subfragments of ScpC

A homologous sequence search identified that *scpC* shares sequence similarity with the cell wall-anchored subtilisin-like serine proteases containing a highly conserved Asp-His-Ser catalytic triad. ScpC is highly similar to the extracellular cell envelope-associated protease, lactocepin of *Lactococcus lactis*. In *Lactococcus*, lactocepin is required for the degradation of casein to make essential amino acids and other peptides. In *S. pyogenes,* its homologue ScpC plays an essential role in evading innate acute inflammatory immune response. Multiple-sequence alignment of the ScpC amino acid sequence with other extracellular cell envelope proteinases indicated that ScpC has a multi-domain structure with similar domain organization as that of other subtilisin-like serine proteases (Hidalgo-Grass et al., 2006). As

described in section 1.3.5.5, ScpC has a predicted N-terminal pre-pro domain (PP) with signal peptide sequence, PR-domain, A-domain, B-domain which is partly fused with the H-domain (B/H), cell wall spacer domain (W) and a cell wall-anchor domain (AN) containing a highly conserved LPXTG motif (Figure 20). The PR-domain is a putative catalytic domain containing the conserved catalytic triad of amino acid residues, Asp^{151}-His^{279}-Ser^{617}.

Each individual domain of proteases often can exist and function independently from the rest of the peptide chain. Therefore, to delineate the minimal functional domain of ScpC required for the IL-8 proteolytic activity, we recombinantly expressed several domains of ScpC in *E. coli* (Figure 21, Table 3.1). The recombinant fusion protein constructs encompassing the N-terminal putative catalytic PR-domain (designated as EC-PR) and PR along with the A-domain of ScpC (designated as EC-PR+A), were expressed using the pQE-60 expression vector with 6xHis as an affinity tag as described in section 2.13.3. The constructs spanning the C-terminal domains including the A-domain along with the B/H-domain (designated as EC- A+B/H) as well as the A-domain alone (designated as EC-A) were expressed as fusion proteins with Glutathione-S-transferase (GST) tag using the pGEX 6P-1 plasmid expression vector. The GST fusion proteins were purified by affinity chromatography (see section 2.13.4), followed by the cleavage of GST from the recombinant protein using a site specific prescission protease. Recombinant expression of the A-domain of ScpC (EC-A) formed insoluble protein aggregates in the form of inclusion bodies, which were observed in the pellet of the *E. coli* cell lysate. In spite of several attempts to solublize the recombinant protein from inclusion bodies, we were unable to purify the soluble recombinant EC-A.

Table 3.1. Recombinant fusion protein constructs of ScpC.

Protein fragments	**Insert**	**Expression Plasmid**	**Host strain**	**Affinity tag**
EC-ScpC	106-1564 aa	pQE-60	M15 [pREP4]	6xHis
EC-PR+A	106-1126 aa	pQE-60	M15 [pREP4]	6xHis
EC-PR	106-689 aa	pQE-60	M15 [pREP4]	6xHis
EC-A+B/H	690-1560 aa	pGEX-6P-1	DH5α	GST
EC-A	690-1127 aa	pGEX-6P-1	DH5α	GST

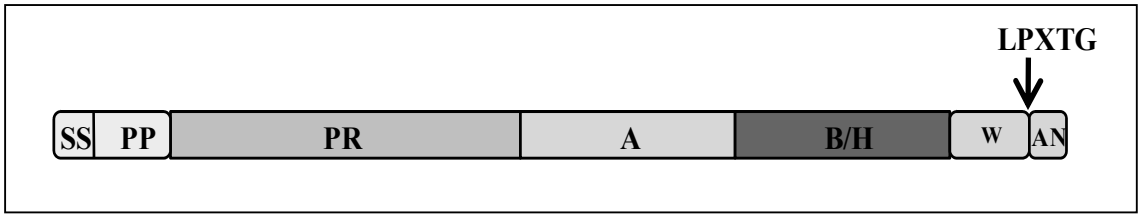


Figure 20. **Schematic representation of the domain organization of ScpC**.

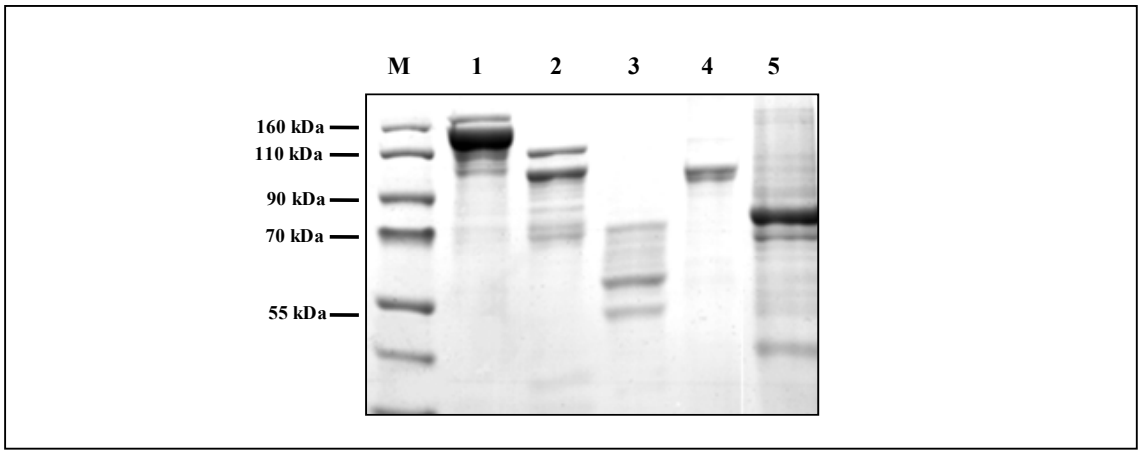


Figure 21. SDS-PAGE analysis showing the bands corresponding to purified recombinant fusion proteins spanning distinct domains of ScpC. Lane 1-5: 1. EC-ScpC, 2. EC-PR+A, 3. EC-PR 4. EC-A+B/H, 5. Cell lysate of *E.coli* containing pQE-60 derivative showing expression of A-domain.

3.1.7 Identification of the minimal essential domain required for the proteolytic activity of ScpC

The minimal domain required for the proteolytic activity of ScpC was evaluated *in vitro* by monitoring the IL-8 degradation by recombinant fusion protein constructs spanning distinct domains of ScpC. For this, recombinant protein constructs of ScpC were incubated with IL-8 for 16 h and the amount of IL-8 was determined by ELISA. By determining residual IL-8, results indicated that the EC-PR, a putative catalytic domain with serine protease activity, was incapable of degrading IL-8 independently. However, a significant degradation of IL-8 by EC-PR+A was observed. In contrast, the recombinant EC-A+B/H encompassing C-terminal domains was unable to degrade IL-8 (Figure 22 A&B). These results indicate that the PR-domain and the flanking A-domain are essential for the proteolytic cleavage of IL-8 and deletion of the C-terminal domains does not affect the proteolytic activity of ScpC protease**.** The role of the A-domain in defining substrate specificity of a cell envelope serine proteinase

PrtP of *Lactococcus lactis* has been shown by Siezen et al. (1993). The protein engineering studies of PrtP showed that two adjacent residues Arg and Lys (747-748) lying in the A-domain are involved in determining the cleavage specificity of PrtP towards caseins and other peptide substrates indicating the potential role of A-domain in defining substrate specificity of the protease. Thus, the observation that the recombinant protein EC-PR+A encompassing the PR-domain along with the A-domain efficiently degrades IL-8, while EC-PR itself could not show proteolytic activity against IL-8, suggests that the A-domain may play a role in regulating the proteolytic activity of ScpC by dictating substrate specificity.

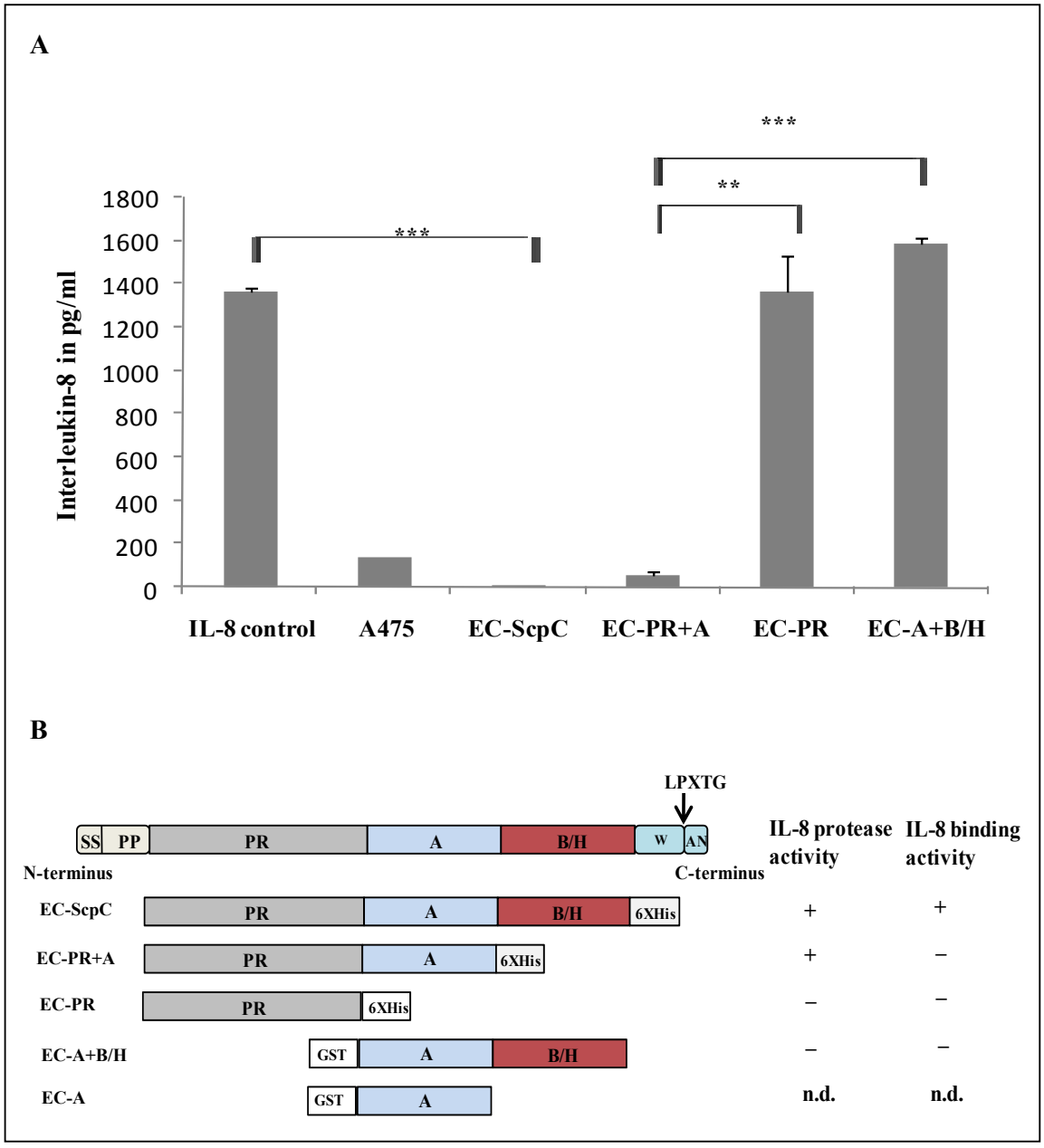


Figure 22. Localization of the domain required for the proteolytic activity of ScpC. A) Histogram representing the IL-8 degrading ability of purified recombinant full-length ScpC and recombinant protein constructs spanning distinct domains of ScpC. The amount of IL-8 was determined after incubation of recombinantly expressed fusion protein or PBS (control) with recombinant IL-8 for 16 h. The supernatant of A475 was taken as a positive control. B) The domain organization of full-length ScpC and schematic representation of the domains of ScpC expressed as recombinant fusion proteins. The IL-8 protease activity and the IL-8 binding ability of fusion protein constructs of ScpC are indicated on the right hand side of the schematic overview of the ScpC domain organization.

3.1.8 Characterization of the IL-8 binding domain of ScpC

The next aim was to identify the minimal ligand binding domain of ScpC. Full-length ScpC and recombinant proteins encompassing distinct domains of ScpC were analysed for the binding of radiolabeled IL-8. By using [^{125}I] IL-8 in the ligand-binding assay, the results indicated that the full-length ScpC (EC-ScpC) in native form showed strong affinity for IL-8 (Figure 23). In contrast, recombinant protein EC-PR+A and EC-PR did not show affinity for IL-8. EC-A+B/H encompassing C-terminal domains showed weak interaction with IL-8, suggesting that full-length ScpC might be required for the interaction with IL-8. Recombinant GST, used as a control, did not show interaction with radiolabeld IL-8. When recombinant full-length ScpC and protein constructs encompassing distinct domains of ScpC were separated on SDS-PAGE gel under denaturing conditions, full-length ScpC lost the ability to interact with IL-8 (data not shown).

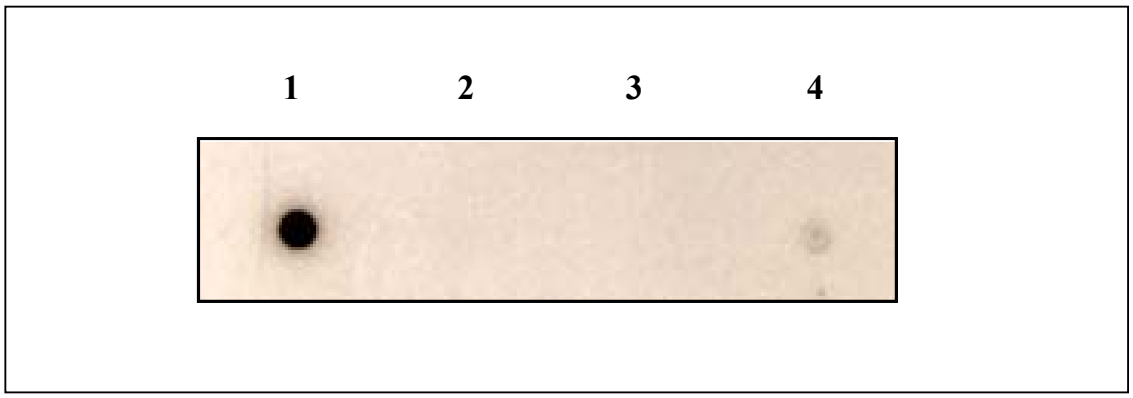


Figure 23. Binding of IL-8 with recombinant ScpC. 5μg of each recombinant protein spanning subfragments of ScpC were loaded on a nitrocellulose membrane and the membrane was probed with radiolabeled [^{125}I] IL-8 ($4x10^5$ c.p.m./ml) for 30 min and autoradiographed overnight. Lane 1. Purified recombinant EC-ScpC, Lane 2. EC-PR+A, Lane 3. EC-PR, Lane 4. EC-A+B/H

3.1.9 Substrate specificity of streptococcal chemokine protease C

For the purpose of identifying an inhibitor for ScpC protease, it was aimed to develop a high throughput assay for measuring the enzymatic activity of ScpC. As mentioned before, ScpC belongs to a family of subtilisin-like serine protease which specifically inactivates IL-8 by a single proteolytic cleavage event (Edwards et al., 2005). It cleaves IL-8 on its C-terminus, at a peptide bond between glutamine and arginine, releasing 13 amino acids of the C-terminus (Figure 24, see section 1.3.5.1).

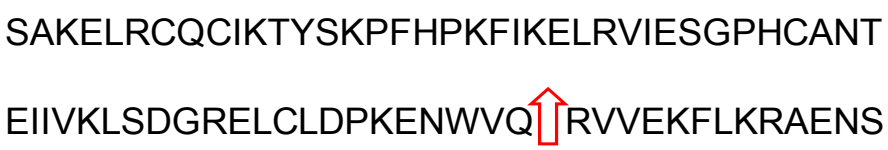
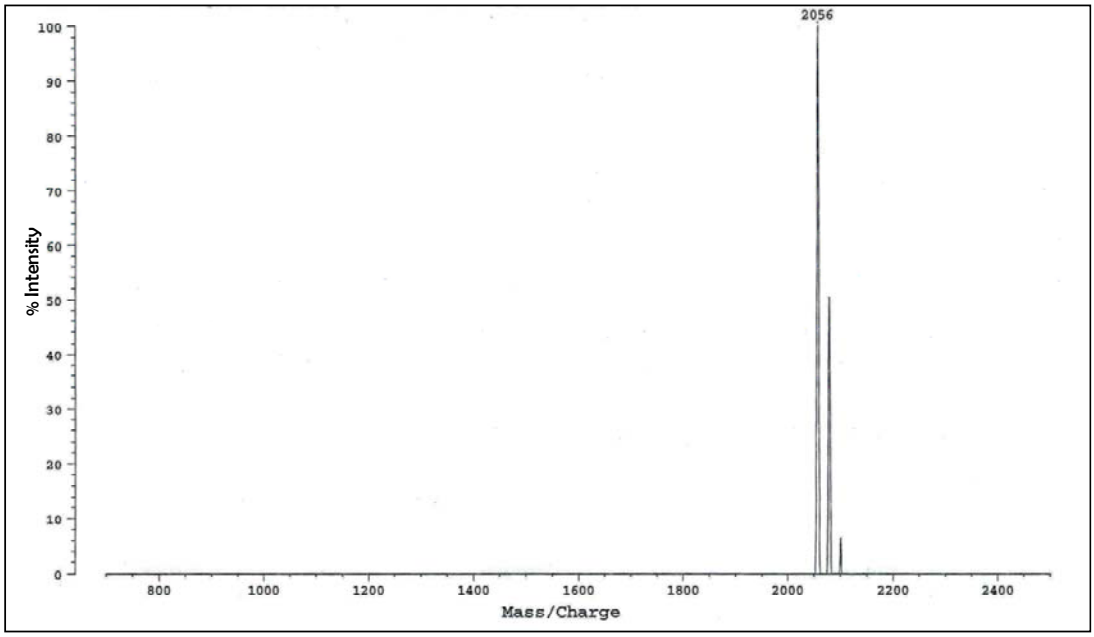


Figure 24. **Amino acid sequence of IL-8**. Arrow indicates the cleavage site of ScpC.

Using the natural substrate, IL-8, is costly and requires a complicated assay (ELISA) which is not suitable for testing a large library of inhibitors. ScpC proteolytic activity has been reported to be inhibited by serine protease inhibitors like Pefabloc, benzamidine and soyabean trypsin inhibitor (Hidalgo-Grass et al., 2004). Based on these findings, it was suggested that ScpC might have a trypsin like activity. Therefore, a panel of commercially available substrates used for assaying trypsin activity, such as Bz-Arg-pNA and Z-Gly-Pro-Arg-pNA, were tested against ScpC. Recombinant ScpC did not cleave these trypsin substrates. Therefore, a chromogenic substrate analogue of the site of cleavage of human IL-8 by ScpC was synthesized. Since proteases which belong to this family do not recognize the sequence at the C-terminal to the bond to be hydrolyzed, therefore the C-terminal to the site of cleavage of IL-8 was replaced by a chromogenic leaving group. Thus, a synthetic chromogenic peptide derivative comprising six amino acid residues corresponding to the amino acids present upstream to the site of cleavage of IL-8, with p-nitroanilide (pNA) present at the C-terminus and glutamine at the P1 positions to the cleavage site (Ac-KENWVQ-pNA) was synthesized and examined for the cleavage by ScpC. Since the amino acid sequence of the substrate around the cleavage site can be a major determining factor for many proteases, a synthetic peptide substrate containing 16 amino acid residues corresponding to eight amino acid residues of IL-8 flanking on the either side of peptide bond to be cleaved by ScpC (Ac-^{52}DPKENWVQRVVEKFLK^{67}-amid) was synthesized. The substrates were separated and purified by high performance liquid chromatography (HPLC) and molecular mass was determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The hydrolysing activity of ScpC against 16-mer synthetic substrate was determined after incubating the substrate for 16-18 h with rScpC as well as with the supernatant of *S. pyogenes* JS95 culture medium, by HPLC and MALDI-MS. These substrates were unable to be cleaved by rScpC and by culture supernatant of *S. pyogenes,* under the conditions tested (Figure 25).

These results suggest that cleavage of IL-8 by ScpC may depend upon the presence of specific amino acid residues at the cleavage site as well as on the overall three dimentional (3D) structural conformation of IL-8.

A



B

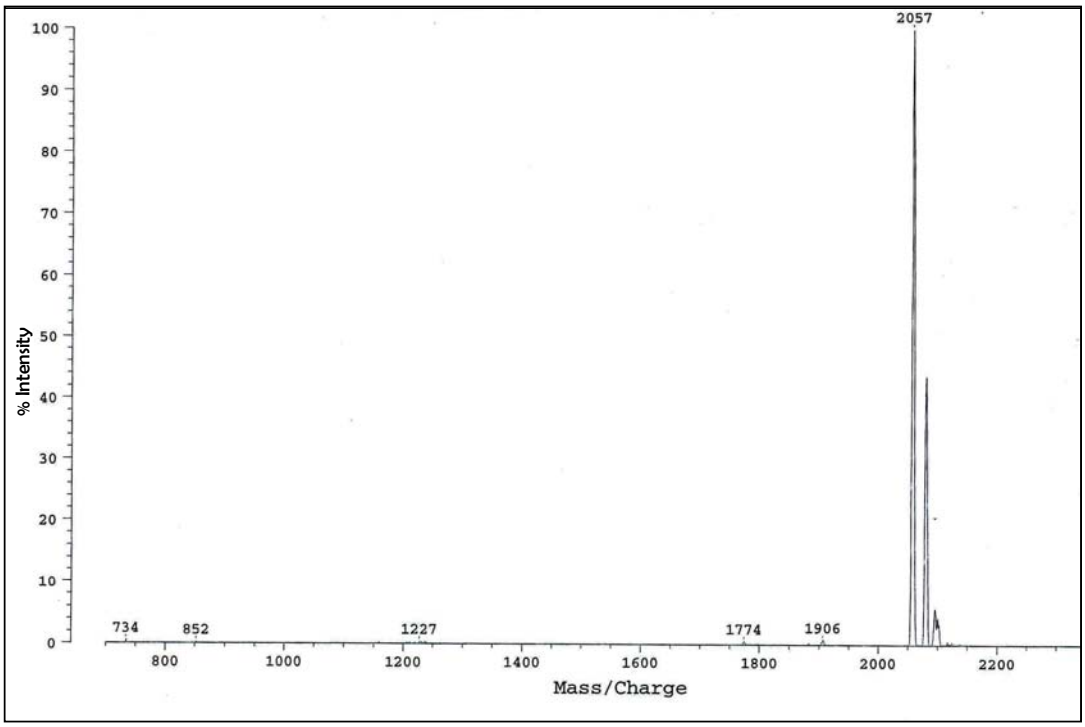


Figure 25. **Mass of synthetic 16-mer peptide containing IL-8 clevage site before (A) and after incubation with recombinant ScpC for 16 h at 37°C**. The reaction products were separated and purified by HPLC and the peptide fragments were analysed by MALDI-MS.

3.2 Role of ScpC in the invasion process of M3 *S. pyogenes* into human endothelial cells

3.2.1 Internalization of recombinant ScpC coated latex beads by primary human umbilical vein endothelial cells (HUVEC)

The endothelial expression of cytokines plays an important role in regulating the inflammatory and immunological response towards infection. Modulating the cytokine production by endothelial cells can contribute significantly to the progression or clearance of the microbial infection via regulating the inflammatory response. Human endothelial cells produce IL-8 constitutively and IL-8 secretion by these cells is induced upon stimulation with proinflammatory cytokines like tumor necrosis factor alpha, IL-1, LPS (Chen and Manning, 1996). Studies have shown that endothelial cells become major producers of IL-8 during bacterial infection in leukopenic cancer patients, with a disease condition where lack of leukocytes was observed (Oude Nijhuis et al., 2003). Also, the airway epithelial cells are capable of releasing inflammatory mediators including IL-1, TNFα, IL-8 and IL-6. The expression and release of IL-8 by A549 lung epithelial cells has been shown to be induced by serine proteinases like trypsin and thrombin (Wang et al., 2006b). These findings prompted us to investigate the *in vitro* interaction of ScpC with HUVEC and A549 lung epithelial cells. The HUVEC primary cell cultures between the second and fifth passage were used for investigating the interaction with ScpC coated polystyrene latex beads. For this, inert latex polystyrene beads (3 μm and 1 μm diameter) were coated with purified recombinant full-length ScpC (ScpC-beads) and incubated with host cells in growth medium containing fetal calf serum for 2 h at 37^{o}C under neutral pH environmental conditions. The cells were rinsed thrice with endothelial cell growth medium (EGM 2 basal medium) and fixed. Samples were then processed for analysis by scanning electron microscopy and immunofluorescence microscopy. ScpC-beads adhered to and were efficiently internalized by HUVEC (Figure 26 A-E), whereas no internalization of bovine serum albumin (BSA) or glutathione S-transferase (GST) coated beads was detected. Internalization of ScpC-beads by HUVEC occurred only by a subpopulation of early passage cells, with some cells showing pronounced uptake of ScpC-beads (Figure 27) indicating that cell specific factors/receptors might play an important role in mediating the interaction of ScpC-beads with HUVEC. In contrast, when interaction of ScpC-beads was tested with A549 lung epithelial cells under neutral pH conditions, the ScpC-beads did not adhere to nor invaded A549 cells. Conclusively, this data shows that ScpC triggers the

uptake of latex beads by primary endothelial cells indicating the potential role of ScpC as an adhesin and invasin which can promote the invasion of *S. pyogenes* into endothelial cells.

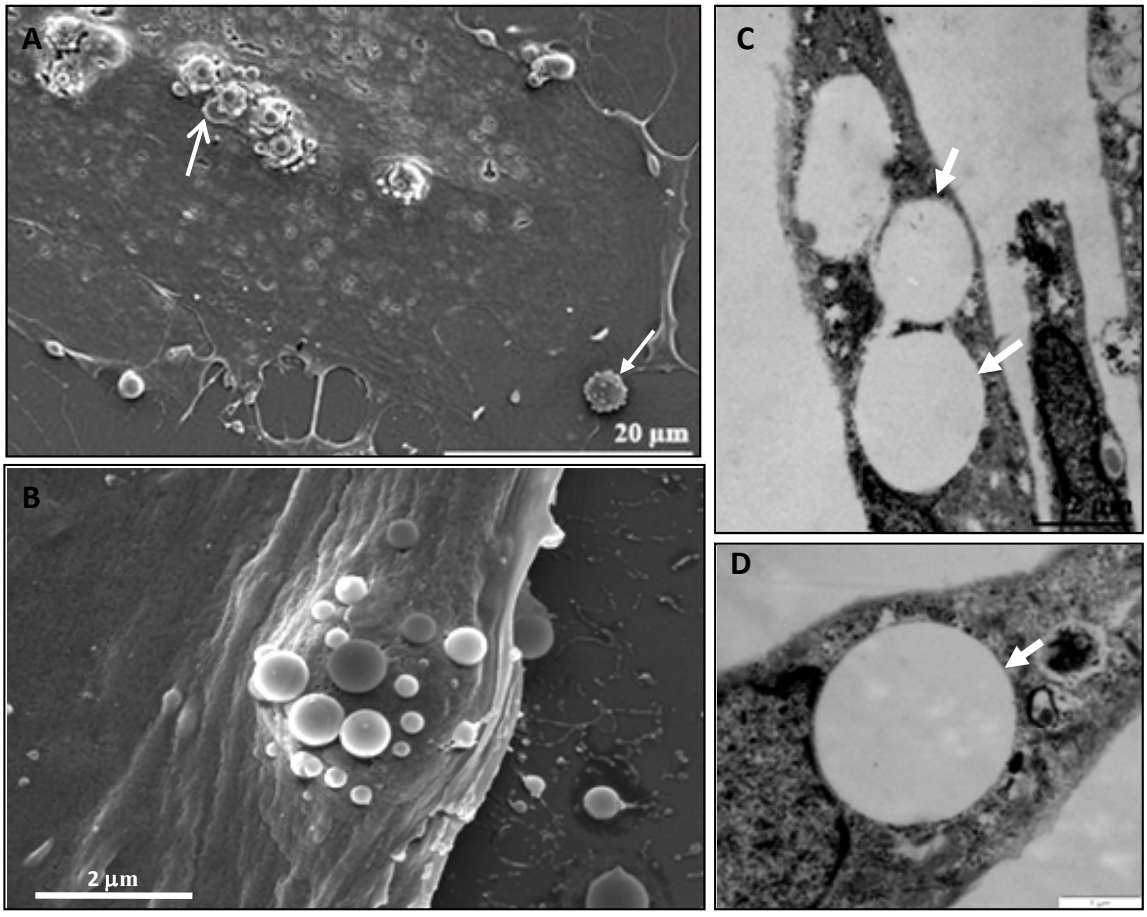


Figure 26a. Electron microscopic images showing the adherence to and internalization of recombinant ScpC coated latex beads by HUVEC. HUVEC were co-incubated with ScpC-beads for 2 h, fixed and processed for scanning electron microscopy (A&B) and transmission electron microscopy (C&D). Arrows indicate adherent and internalized ScpC-beads (A). C&D) Transmission electron microscopy of ultrathin images showing intracellular ScpC-beads. Arrows delineate the intracellular ScpC-beads.

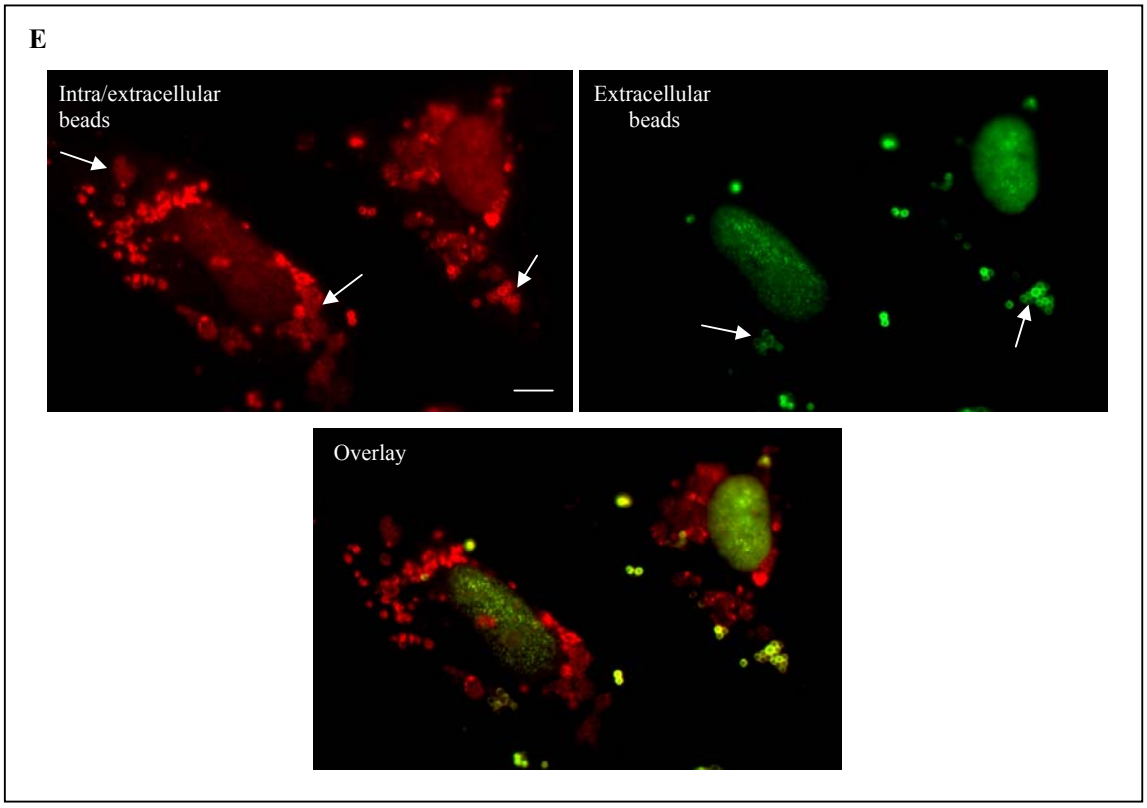


Figure 26b. Microscopic images showing the adherence to and internalization of recombinant ScpC coated latex beads by HUVEC. E) Internalization of ScpC-beads by HUVEC is shown by immunofluorescene microscopy. HUVEC were co-incubated with ScpC-beads (1 μm diameter) in a ratio of approximately 100:1 for 2 h. Immunostaining was performed to distinguish extracellular beads from intracellular beads. Cells were incubated for 1 h with rabbit polyclonal anti-ScpC IgG and then with anti-rabbit fluoro® 488 conjugated antibody. Cells were permeablised with triton-X for 5 min and then incubated again with anti-ScpC IgG. The anti-rabbit fluoro® 568 conjugated antibody was then used as secondary antibody. Intracellular latex beads appear red and extracellular beads are labeled green.

3.2.2 Co-localization of ScpC with LAMP-1

Lysosomes are membrane bound compartments of the cells which have traditionally been considered as terminal degradative compartments. Various studies have shown that *S. pyogenes* can successfully escape the intracellular lysosomal killing via various mechanisms including inhibition of the fusion of phagosome with lysosomes, escape from phagosome into the cytoplasm and by enhancing survival inside the phagolysosome (Medina et al., 2003; Staali et al., 2006). To identify the intracellular fate of internalized beads, we tested if ScpC-beads follow the classical cellular endocytic pathway. For this, we examined the intracellular trafficking of ScpC-beads by immunostaining the intracellular compartments with an antibody against lysosomal-associated membrane protein 1 (LAMP-1), a well known marker for late

endosomes/lysosomes and examined the co-localization of ScpC-beads with these cellular compartments. The immunofluorescence microscopic analysis revealed the presence of internalized ScpC-beads in LAMP-1 positive compartments within the cells after incubation of ScpC-beads with HUVEC for 2 h (Figure 27). This shows that the internalized ScpC-beads eventually fused with the lysosomes, thus indicating that ScpC can trigger the internalization process and subsequent targeting of ScpC carriers to the lysosomes in the endothelial cells.

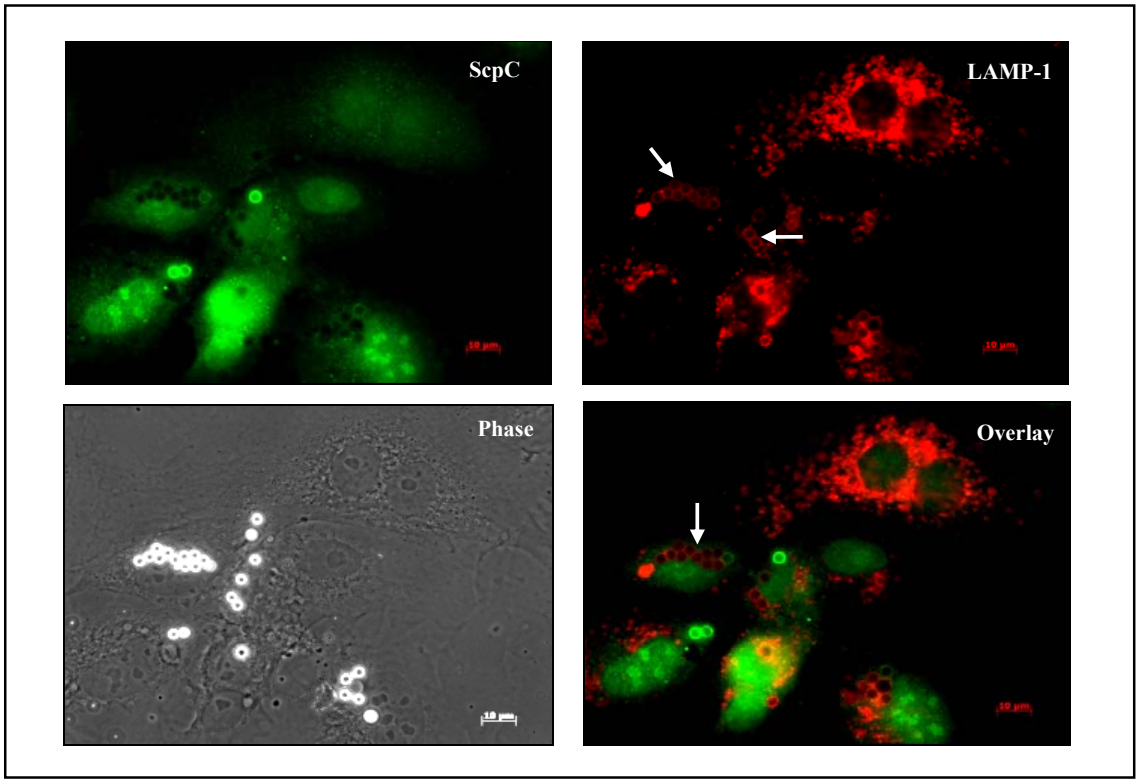


Figure 27. Co-localization of ScpC-beads with LAMP-1 positive compartments. HUVEC were incubated with ScpC-beads for 2 h. After the incubation period, cells were rinsed with EGM 2 basal medium and fixed. Beads were immunolabeled with rabbit polyclonal anti-ScpC antibody. Anti-rabbit Alexa fluor® 488 conjugated antibody was used to visualize the beads (green). To stain lysosomes, treated cells were immunolabeled with mouse monoclonal antibody against lysosome associated membrane protein 1 (LAMP-1). Arrows indicate intracellular ScpC-beads residing in the LAMP-1 positive compartments. Then, an anti-mouse fluorphore conjugated antibody was used to detect lysosomal compartments (red). Each bar represents 10 µm.

3.2.3 Role of N-terminal PR domain in the internalization of latex beads by HUVEC

In addition to the above described experiments, purified recombinant ScpC subfragments, as described in section 3.1.6.1, were evaluated for their interaction with HUVEC. For this purpose, the latex beads were coated overnight at 4°C with purified recombinant fusion

proteins spanning distinct ScpC domains. The recombinant protein coated beads were then incubated with HUVEC for 2 h. After the incubation period cells were rinsed with EGM 2 basal medium to remove the unbound beads and immunostained for fluorescence microscopic analysis. Latex beads coated with recombinant protein EC-PR, containing putative catalytic domain, were efficiently internalized by HUVEC and subsequently co-localized with LAMP-1 positive compartments (Figure 28B), whereas beads coated with EC-A+B/H rarely adhered to or invaded HUVEC. The latex beads coated with GST protein, which was taken as a control, did not adhere to or internalized by the endothelial cells (Figure 28C)**.** The internalization of EC-PR coated beads by HUVEC was comparable to the uptake of full-length ScpC coated beads (Figure 28 A) indicating that the N-terminal PR-domain of ScpC is sufficient to trigger the cellular uptake and subsequent fusion of latex beads with lysosomes. Thus, by identifying the domain involved in the invasion mediating activity of ScpC it was possible to narrow down the sequence essential for the invasion associated function of this large surface protein.

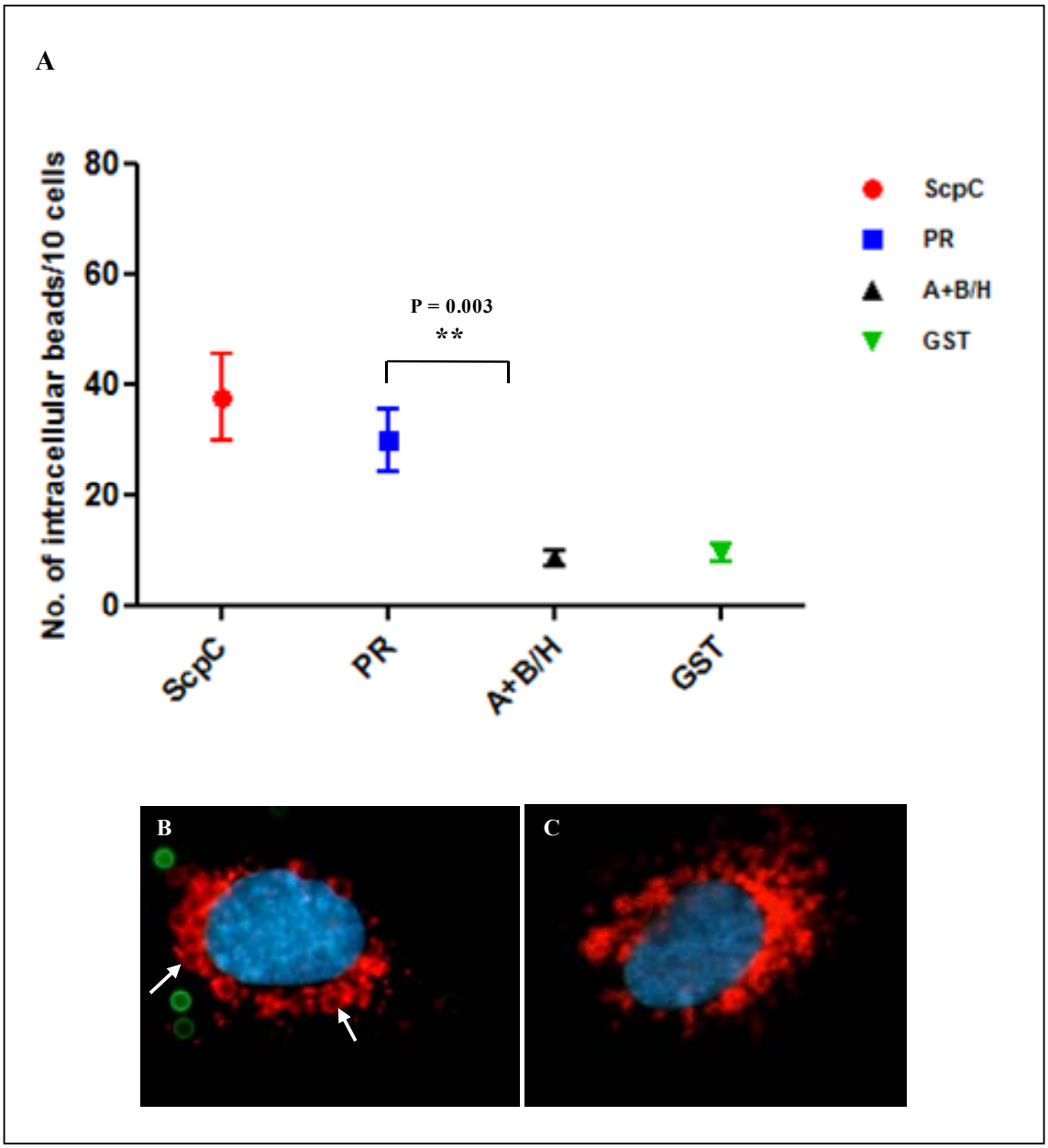


Figure 28. The N-terminal PR-domain is sufficient to trigger the uptake of latex beads and their fusion with lysosomes. Latex beads were coated overnight at 4ºC with purified recombinant full-length ScpC and recombinant fusion proteins encompassing subfragments of ScpC. Recombinant protein coated beads were incubated with HUVEC for 2 h at 37°C and fixed. HUVEC incubated with protein coated beads were immuno-stained using anti-ScpC antibody and anti-rabbit Alexa fluor® 488 (green) conjugated secondary antibody. The lysosomes were stained by using mouse monoclonal antibody against LAMP-1. LAMP-1 positive compartments were visualized by using anti-mouse Alexa fluor® 568 (red) antibody. Intracellular beads fused with lysosomes were counted for each fusion protein construct. Glutathione S-tranferase (GST) was purified and used as a negative control. Data is based on the analysis of 60 cells for each fusion protein construct. B) The primary culture HUVEC were incubated with EC-PR-beads and stained with similar procedure as described above. C) Control beads coated with GST did not adhere to nor internalized by HUVEC.

3.2.4 Interaction of purified recombinant ScpC with HUVEC

The next aim was to analyse whether cultured endothelial cells can internalize purified rScpC without prior coating on the latex beads. For this, HUVEC were incubated with purified rScpC (20 µg/ml) for 2 h at 37°C in a humidified environment containing 5% CO_2. After the incubation period, cells were rinsed three times to remove unbound rScpC and fixed. Consistent with the data showing the adherence and uptake of ScpC-beads by HUVEC, purified rScpC bound to HUVEC. The binding of rScpC with endothelial cells occurred in a wide concentration range (10-100 µg/ml) and was highest at 2 h post-incubation (Figure 29). A preliminary analysis of *in vitro* interaction of ScpC with HUVEC indicates that ScpC triggers cellular responses including disruption of membrane and intercellular junctions of endothelial cells.

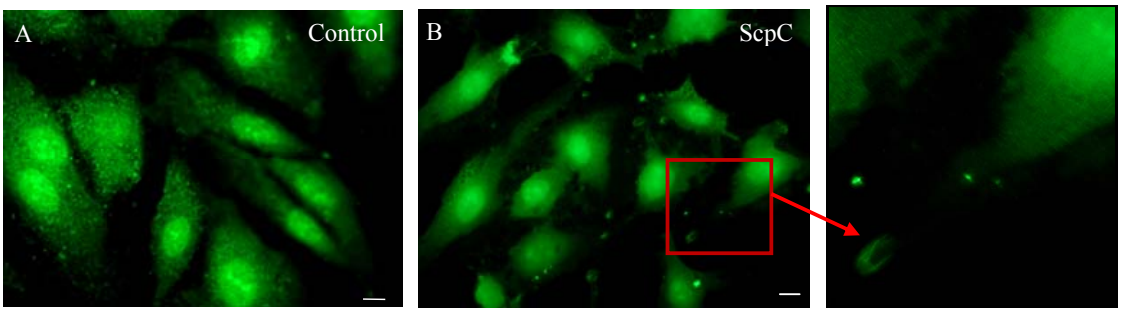


Figure 29. Interaction of recombinant ScpC with HUVEC. HUVEC were incubated with purified rScpC for 2 h at 37°C. Cells were then washed three times with EGM 2 to remove unbound rScpC. The cells were fixed and stained for immuno-fluorescence microscopic analysis with rabbit polyclonal anti-ScpC IgG. An anti-rabbit Alexa fluor® 488 (green) conjugated antibody was used to visualize the interaction of rScpC with HUVEC. Enlarged area indicates the association of rScpC with the endothelial cell edges. Bar represent 10µm.

3.2.5 Acidic pH triggers the interaction of ScpC with host cells

It has been previously shown that exocytosis of lysosomes is triggered in the cells in response to the membrane damage in order to reseal the damaged areas on the membrane as a plasma membrane repair mechanism (Gerasimenko et al., 2001; Reddy et al., 2001; Wessels et al., 1991). Therefore, the acidic pH (pH 6.3) environmental condition for host cells was tested in order to mimic the microenvironment of an *in vivo* infection, where host cells may encounter acidic conditions during *S. pyogenes* infections due to enhanced lysosomal exocytosis caused by secreted or cell bound proteins of *S. pyogenes*. For this, ScpC-beads were incubated with A549 lung epithelial cells in acidified environment (pH 6.3) at 37°C. Under acidified

extracellular conditions, ScpC-beads strongly adhered to A549 cells at 2 h post-incubation but were not internalized (Figure 30).

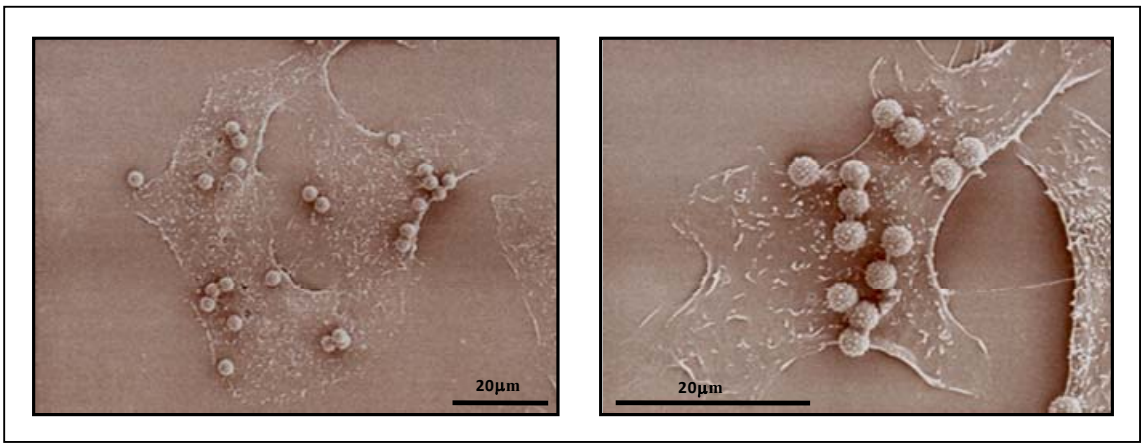


Figure 30. Adherence of ScpC-beads to A549 lung epithelial cells. ScpC-beads were incubated with A549 lung epithelial cells for 2 h in DMEM and citric phosphate buffer to provide extracellular pH of 6.3. After 2 h of incubation with ScpC-beads, A549 cells were fixed and processed for scanning electron microscopy. ScpC-beads efficiently adhered to A549 cells under low pH conditions.

It is noteworthy that ScpC-beads did not adhere to nor internalized by primary HUVEC at 6th passage and later. This might be due to the loss of the specific receptors by HUVEC which were involved in the interaction with ScpC**.** This data further suggests that cell specific factors/receptors are involved in the process of internalization of ScpC-beads by HUVEC. However, when HUVEC at 6th passage were incubated with ScpC-beads under acidic pH (pH 6.3) environment conditions, ScpC-beads efficiently attached to and internalized by cells indicating that low pH environmental conditions triggers the adherence to and internalization of ScpC-beads by endothelial cells (Figure 31). These data suggest that acidic pH extracellular environmental conditions might have an impact on the ScpC-mediated invasion of *S. pyogenes* into host cells.

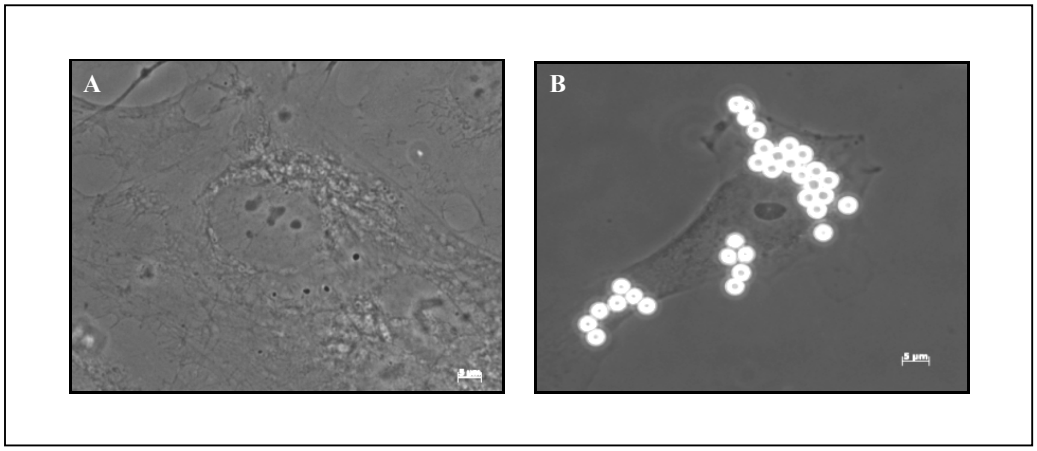


Figure 31. Acidic pH triggers the adherence of ScpC-beads to HUVEC. HUVEC at 6th passage were incubated with rScpC coated latex beads for 2 h under neutral (A) or acidic pH environment (B). ScpC-beads showed adherence to HUVEC when exposed to low pH (pH 6.3). Whereas under neutral pH, ScpC-beads did not adhere nor internalized by HUVEC. Bar corresponds to 5 μm.

3.2.6 Effect of specific antibodies raised against ScpC on its functional activity

3.2.6.1 Neutralizing effect of anti-ScpC antibody towards the internalization of ScpC coated polystyrene latex beads by HUVEC

Rodriguez-Ortega et al. (2006) have shown that immunization with ScpC had a protective effect against *S. pyogenes* infection after mucosal challenge in a murine model of infection. Another report has shown that the human serum collected from patients with *S. pyogenes* infections contain antibody titer against ScpC, thus indicating the immunogenicity of ScpC in humans (Lei et al., 2000). Based on these reports, we next investigated the effect of specific antibodies induced against rScpC on the internalization of ScpC-beads by HUVEC. The ScpC-beads were pretreated with rabbit polyclonal anti-ScpC IgG for 1 h at room temperature before the co-incubation with HUVEC. Treatment of ScpC-beads with anti-ScpC antibodies almost completely abolished the attachment to and internalization of ScpC-beads by HUVEC. Non-immune rabbit IgG, taken as negative control, did not affect the adherence to or internalization of ScpC-beads by HUVEC (Figure 32). This indicated that the internalization of ScpC-beads by HUVEC is due to the specific interaction between ScpC and endothelial cells and not because of a non-specific association of latex beads with HUVEC. These results suggest that antibodies induced against ScpC may show an inhibitory effect on the invasion of *S. pyogenes* into endothelial cells via blocking the invasion mediating activity of ScpC.

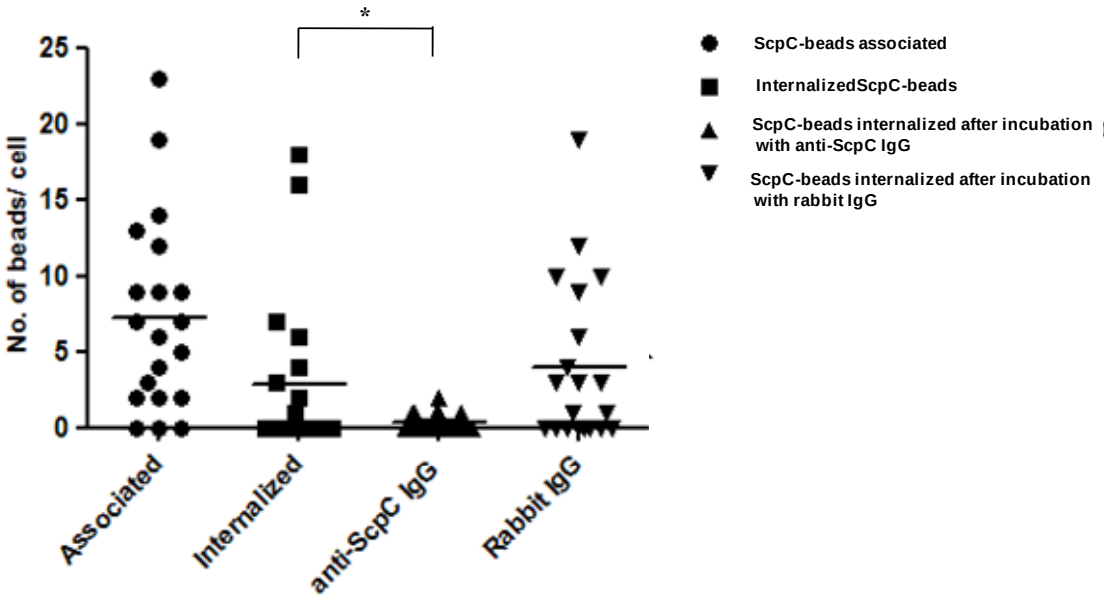


Figure 32. Inhibition of invasion of ScpC coated latex beads into HUVEC by anti-ScpC antibodies. The ScpC-beads were incubated with rabbit polyclonal anti-ScpC antibodies for 1 h and then co-incubated with HUVEC for 2 h. Pre-incubation of ScpC-beads with anti-ScpC IgG completely abolished the adherence to and invasion of ScpC-beads into HUVEC. Non-immune rabbit IgG served as a control. Each dot represents the total number of ScpC-beads associated with or internalized by one cell. Horizontal bars represent mean.

3.2.6.2 Effect of anti-ScpC antibody on the proteolytic activity of ScpC

We next investigated if ScpC specific IgG have a neutralizing effect towards the IL-8 proteolytic activity of ScpC. The culture supernatant of *S. pyogenes* strain A475 and its isogenic Δ*scpC* mutant was pretreated with rabbit polyclonal anti-ScpC IgG and then co-incubated with IL-8. The effect of anti-ScpC IgG on the IL-8 proteolytic activity of purified rScpC was determined as well, by incubating rScpC with anti-ScpC IgG for 1 h prior to co-incubation with IL-8. The amount of residual IL-8 was measured by cytokine ELISA. In contrast to the blocking effect of anti-ScpC IgG on ScpC-mediated uptake of latex beads by endothelial cells, the pre-incubation of rScpC with anti-ScpC IgG had no effect on its proteolytic activity. Also, the IL-8 proteolytic activity of culture supernatant of A475 and its isogenic Δ*scpC* mutant remained unaltered after co-incubation with anti-ScpC IgG (Figure 33). Taken together, these results show that anti-ScpC antibody can specifically block the ScpC-mediated invasion of latex beads into endothelial cells without altering its proteolytic activity. Therefore, it may be anticipated that antibodies raised against ScpC might have a

protective role against *S. pyogenes* infection via inhibiting the ScpC-mediated interaction of *S. pyogenes* with endothelial cells. Additionally, the specificity of antibody for neutralizing the invasion mediating activity but not the proteolytic activity of ScpC suggests that different epitopes are involved in these two distinct functions of ScpC.

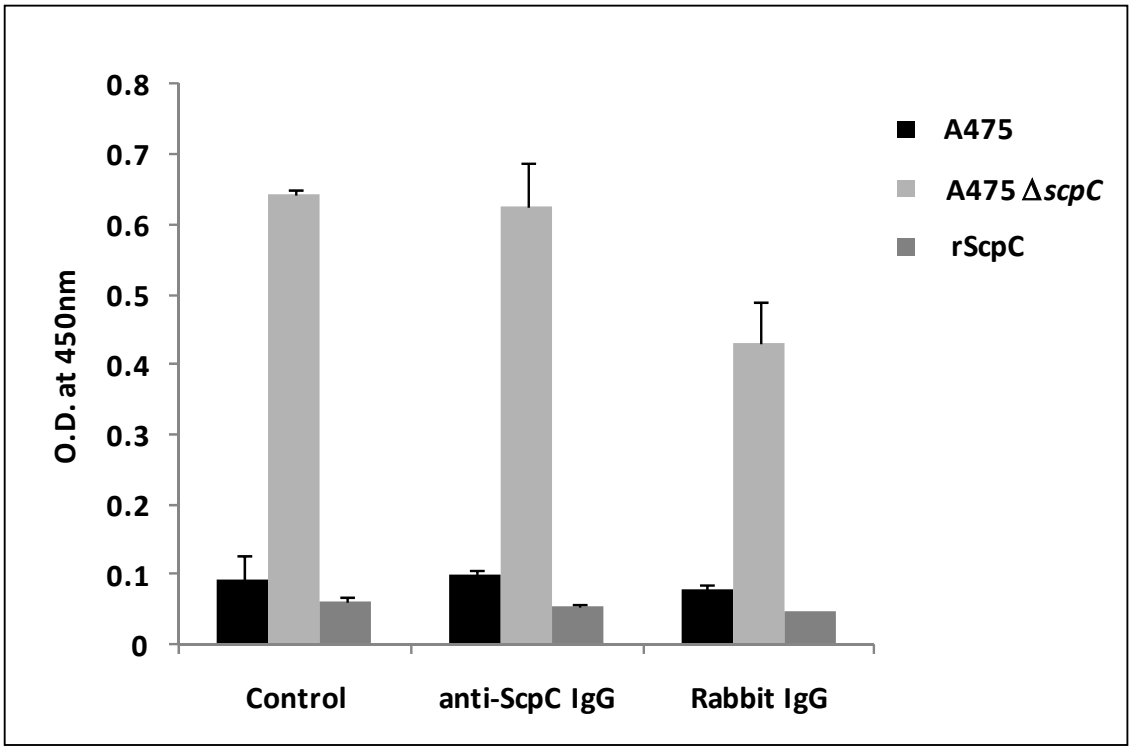


Figure 33. **Effect of anti-ScpC antibody on the IL-8 proteolytic activity of ScpC.** The culture supernatant of *S. pyogenes* strain A475 and its isogenic Δ*scpC* mutant were incubated with rabbit polyclonal anti-ScpC IgG or with preimmune rabbit IgG for 1 h, prior to the incubation with IL-8 to determine the IL-8 proteolytic acitivty of ScpC as decribed above. To test if the specific antibodies raised against ScpC can block the proteolytic activity of rScpC, the purified rScpC was pre-incubated with anti-ScpC IgG and then incubated with IL-8 to determine the proteolytic activity of ScpC. Each bar represents mean of optical density ±SD.

3.3 *In vitro* invasion of *S. pyogenes* into human umbilical vein endothelial cells

3.3.1 ScpC affects invasion and transcytosis of M3 *S. pyogenes* through HUVEC

Previous studies have shown that M3 *S. pyogenes* can efficiently adhere to and invade primary human endothelial cells. M3 *S. pyogenes* invades endothelial cells via inducing the generation of host cell membrane protrusions around the streptococci that extend to engulf the bacteria, a

process which requires the reorganization of the actin cytoskeleton. Following the internalization into HUVEC, M3 *S. pyogenes* were observed to be localized within phagosomes which subsequently fused with lysosomes (Talay et al., unpublished; N. Peterman, Diploma thesis, 2003). Based on the *in vitro* interaction of ScpC with HUVEC, we examined if ScpC has a direct role in mediating the invasion of *S. pyogenes* into primary human endothelial cells. For this, the invasion of WT A475 and its isogenic Δ*scpC* mutant into a cultured semi-confluent monolayer of HUVEC was compared. The cells were incubated with bacteria for 30 min at a multiplicity of infection (MOI) of 16:1. After the incubation period, cells were rinsed with EGM 2 basal medium and fixed. For studying the invasion mechanism of *S. pyogenes* into HUVEC at later time points, cells were rinsed two times with endothelial cell basal medium at 30 min post-infection to eliminate extracellular bacteria, and incubated further for 30 min and 90 min in EGM 2 supplemented with 2% fetal calf serum. Immunostaining was performed to distinguish intracellular and extracellular bacteria. Analysing the invasion of WT A475 and its isogenic Δ*scpC* mutant into HUVEC at early time points (30 min post-infection), showed a significant reduction in the invasion of Δ*scpC* mutant in comparison to WT A475 (Figure 34). These data further corroborate the previous results showing the ability of recombinant ScpC to trigger the invasion of latex beads into HUVEC. Thus, these results indicate the significant contribution of ScpC in mediating the invasion of M3 *S. pyogenes* into HUVEC.

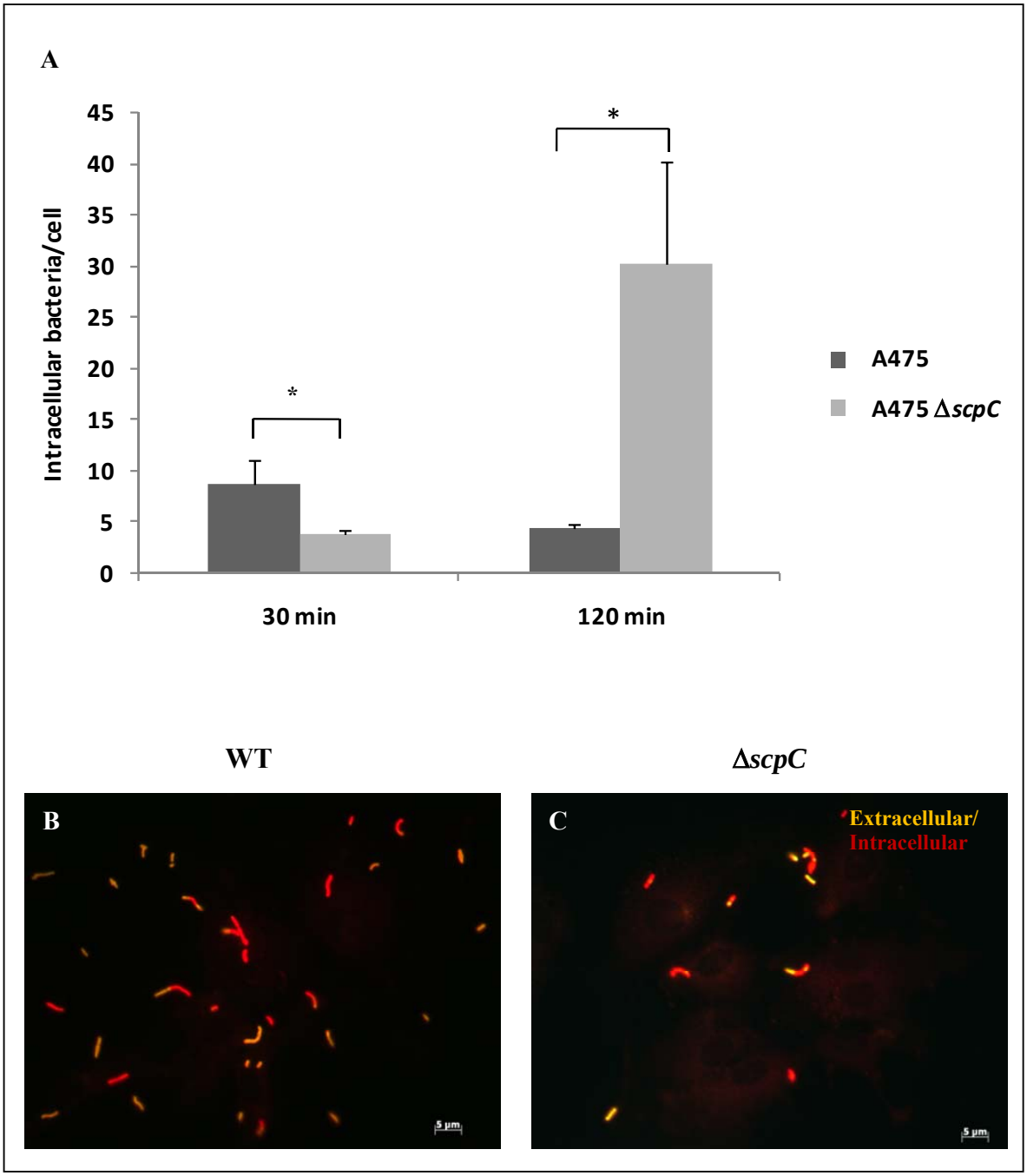


Figure 34. Invasion of WT *S. pyogenes* A475 and its isogenic Δ*scpC* mutant into HUVEC. A) Cells were infected for 30 min with bacteria to cell ratio of 16:1 at 37°C in a humidified atmosphere containing 5% CO_2 and fixed. For studying invasion at later time points, cells were rinsed two times with EGM 2 basal medium at 30 min post-infection to remove extracellular bacteria and incubated further for 90 min in EGM 2 + 2% fetal calf serum. Cells were immunostained to distinguish intracellular bacteria from extracellular bacteria in order to compare the invasion of WT A475 and its isogenic Δ*scpC* mutant into HUVEC. Intracellular bacteria were counted for each cell**.** Representative data is based on the analysis of 25-30 cells for each experiment. B&C). Microscopic images showing the invasion of WT A475 and its isogenic Δ*scpC* mutant strain into HUVEC at 30 min post-infection. Intracellular bacteria appear red and extracellular appear yellow.

However, at later time points (2 h post-infection), significantly (P=0.014) higher numbers of intracellular A475 Δ*scpC* mutant bacteria were observed in comparison to WT A475 (Figure 34A). The number of intracellular WT A475 decreased at 2 h post-infection whereas, A475Δ*scpC* were present in large aggregates (Figure 35) inside a subpopulation of HUVEC at 2 h post-infection. Previous studies of Talay et al. (unpublished) indicated that intracellular M3 *S. pyogenes* can promote its subsequent transcytosis through cultured human endothelial cells (HUVEC) via exocytosis of lysosomes as shown by the presence of LAMP-1 on the surface of HUVEC, closely associated with *S. pyogenes*. Based on these findings, the present data indicate that A475Δ*scpC* mutant lacks the ability to escape from endothelial cells which results in its intracellular accumulation, suggesting that ScpC might play an important role in mediating the transcytosis of *S. pyogenes* across HUVEC.

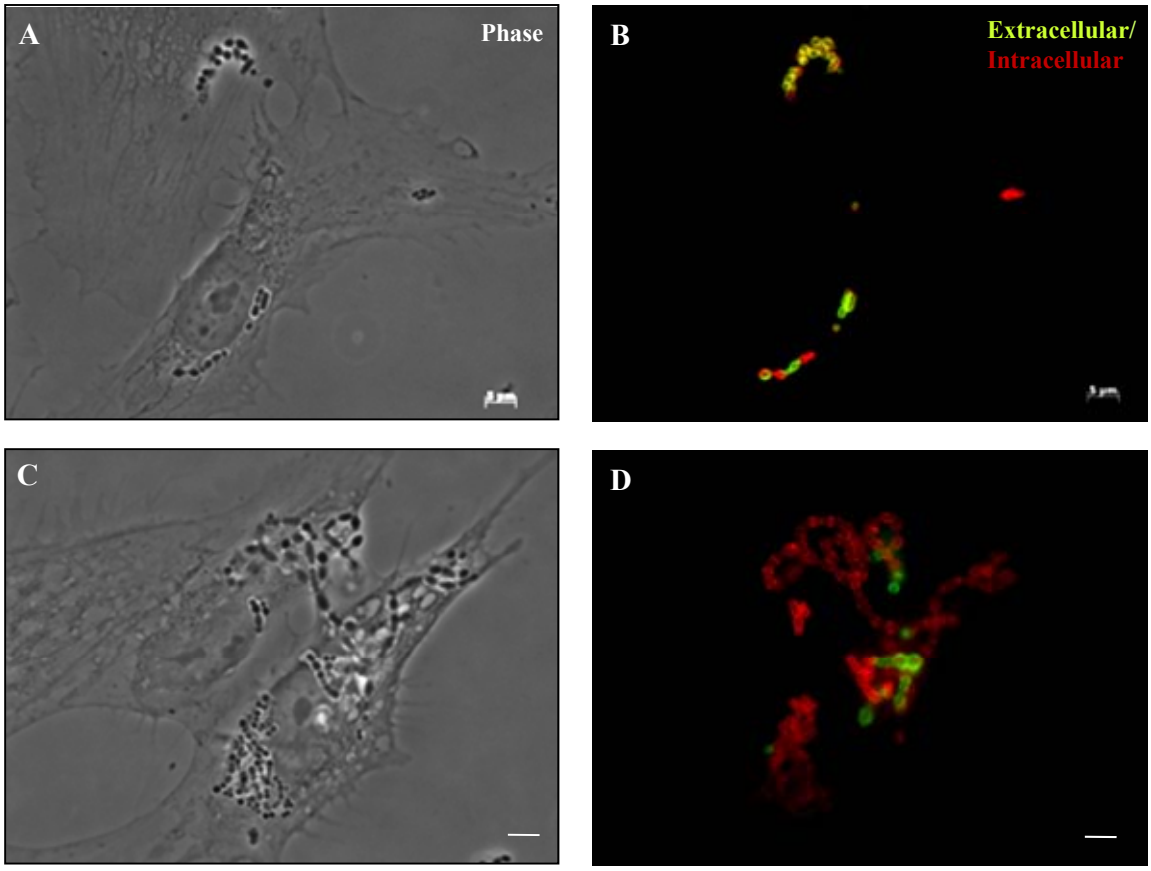


Figure 35. Intracellular accumulation of A475 Δ*scpC* mutant at 2 h post-infection. Cells were infected with A475 (A&B) and isogenic Δ*scpC* mutant (C&D) for 30 min with bacteria to cell ratio of 16:1 and rinsed two times with endothelial cell growth medium to remove extracellular bacteria. Cells were then incubated further for 90 min in EGM 2 supplemented with 2% fetal calf serum at 37°C. The infected cells were immunostained to distinguish intracellular and extracellular bacteria as described in materials and methods. Intracellular bacteria are shown in red and extracellular in green. Each bar indicates 5 μm.

3.3.2 Invasion and intracellular survival of *scpC* mutant *S. pyogenes*

An antibiotic protection assay was conducted to determine the capability of *S. pyogenes* A475 and its isogenic Δ*scpC* mutant to invade endothelial cells. HUVEC were infected for 2 h at MOI of 10:1 in the presence of serum. For the quantification of intracellular bacteria, cells were further treated with gentamicin (100 μg/ml) and penicillin (5 μg/ml) for 2 h to kill the extracellular adherent bacteria (see section 2.22.5). The monolayer of cells was lysed with triton X-100 and cell lysate was plated on blood agar plates. The intracellular viable *S. pyogenes* were quantified by counting colony forming units. In this case, significant reduction (P=0.002) in the intracellular invasion of A475 Δ*scpC* mutant was observed in comparison to

WT parental strain at 2 h post-infection (Figure 36; Table 3.2). It is likely that the reduction in the invasion is due to the reduced survival of the mutant strain instead of the difference in invasion efficiency since previous results indicated significantly higher number of intracellular Δ*scpC* mutant bacteria in comparison to WT A475 at 2 h post-infection. The reduced intracellular survival of Δ*scpC* mutant in comparison to WT A475 can be explained on the basis of intracellular killing of bacteria by bactericidal activity of lysosomes due to the lack of the ability of Δ*scpC* mutant to escape the lysosomal killing, in contrast to WT A475 which is able to escape endothelial cells via exocytosis. Another possibility is that the enhanced endothelial cell membrane injury by the A475 Δ*scpC* mutant in subpopulation of cells might have caused the leakage of antibiotics into the cells, thus killing the intracellular residing Δ*scpC* mutant bacteria. Alternatively, the accumulation of A475 Δ*scpC* mutant inside the cells might have caused the enhanced endothelial cell death which correlates with the previous reports showing the endothelial cell death following the intracellular invasion by *S. aureus* (Menzies and Kourteva, 1998).

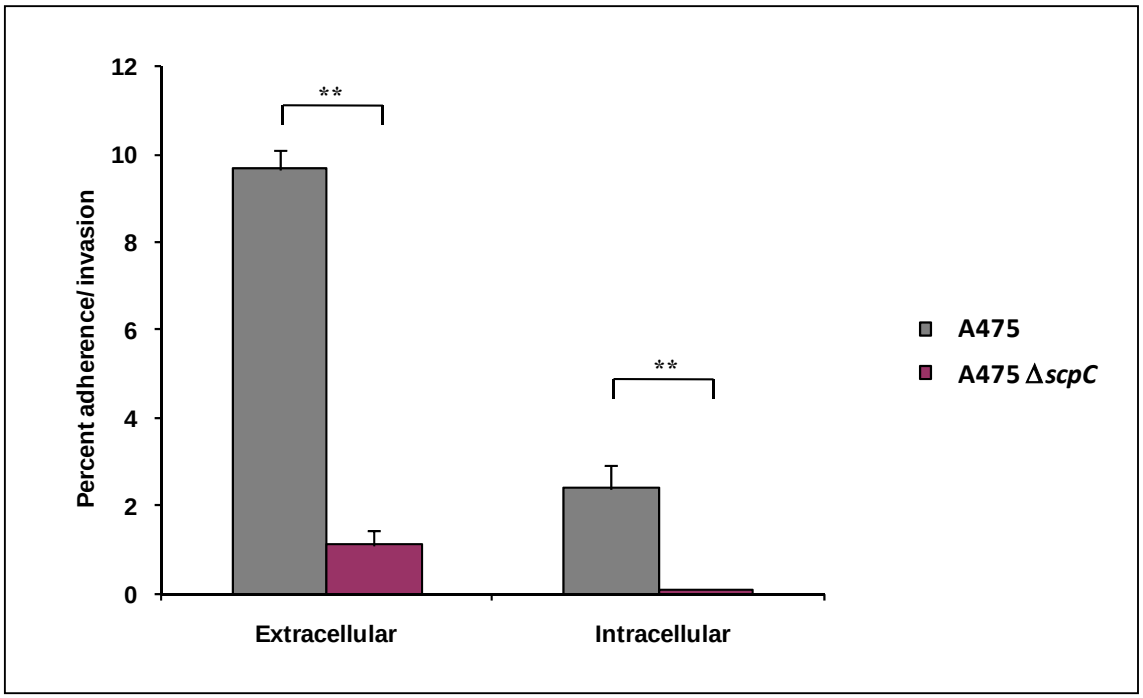


Figure 36. Quantitative analysis of adherence to and invasion of WT A475 and isogenic Δ*scpC* mutant into HUVEC. The cells were infected for 2 h in humidified environment containing 5% CO_2. For the quantification of intracellular bacteria, the cells were additionally treated with antibiotics for another 2 h to kill extracellular bacteria. The cells were then lysed with triton-X 100 and intracellular bacteria were plated on blood agar plates. The colony forming units (cfu) released by cells were counted on the next day and the percentage of invasion was calculated in comparison to the initial inoculum of bacteria. Data is representative of three experiments with each experiment performed in triplicates.

Table 3.2. Number of viable intracellular bacteria per well after infecting HUVEC monolayer for 2 h

S. pyogenes	**cfu/well**	**SD**
WT A475	1.51×10^4	± 0.3482
A475 Δ*scpC*	1.34×10^3	± 2.0450

3.3.3 Characterization of the invasion process of M3 *S. pyogenes* A475 and its isogenic *scpC* mutant

Based on the observed difference in the invasion of WT A475 and its isogenic Δ*scpC* mutant into HUVEC, the invasion process of WT and mutant strain was further characterized. To test whether intracellular bacteria reside in the phagosomal compartment, we aimed to identify the intracellular compartment in which WT A475 and its isogenic Δ*scpC* mutant resides. HUVEC were infected with bacteria to cell ratio of 16:1. After an incubation period of 30 min, cells were washed twice with EGM 2 basal medium to remove extracellular bacteria and then incubated further in EGM 2 supplemented with 2 % FCS at 37^{o}C. After a pre-determined incubation period (30 min, 90 min and 120 min) cells were washed three times with EGM 2 basal medium and fixed. Bacterial cells were labeled with rabbit polyclonal anti-*S. pyogenes* antibody and host cell lysosomal compartments were identified by immuno-staining with monoclonal antibodies against LAMP-1. Immunofluorescence microscopic analysis revealed that following invasion into HUVEC, WT A475 was found in a phagosome-like compartment and most of bacteria co-localized with LAMP-1 at 1 h post-infection indicating that *S. pyogenes* containing vacuoles fused with late endosomes/lysosomes. At 2 h post infection, WT bacteria were identified in LAMP-1 positive compartments, however very less intracellular bacteria could be detected at this time point of infection. At 1 h post-infection, the Δ*scpC* mutant showed co-localization with LAMP-1 indicating that the mutant strain also resides in late endosomes/lysosomes. In contrast to the invasion by WT A475 which showed few numbers of bacteria co-localized with LAMP-1 at 2 h post-infection (Figure 37A), large aggregates of A475 Δ*scpC* mutant showed co-localization with LAMP-1 positive compartments (Figure 37B). This finding shows correlation with our previous results showing

the reduced survival of A475 Δ*scpC* mutant in endothelial cells in comparison to WT parental strain by antibiotic protection assay. Based on these observations, it can only be speculated that the A475 Δ*scpC* mutant might have been partially killed in the lysosomes due to the lack of ability to escape from endothelial cells. However, further studies are required to fully understand the mechanism of ScpC-mediated invasion and transcytosis of *S. pyogenes* across endothelial cells. Taken together, these results indicate that ScpC plays an important role in the invasion process of M3 *S. pyogenes* into endothelial cells which might significantly contribute to enhance the virulence of *S. pyogenes*, enabling them to cause invasive diseases.

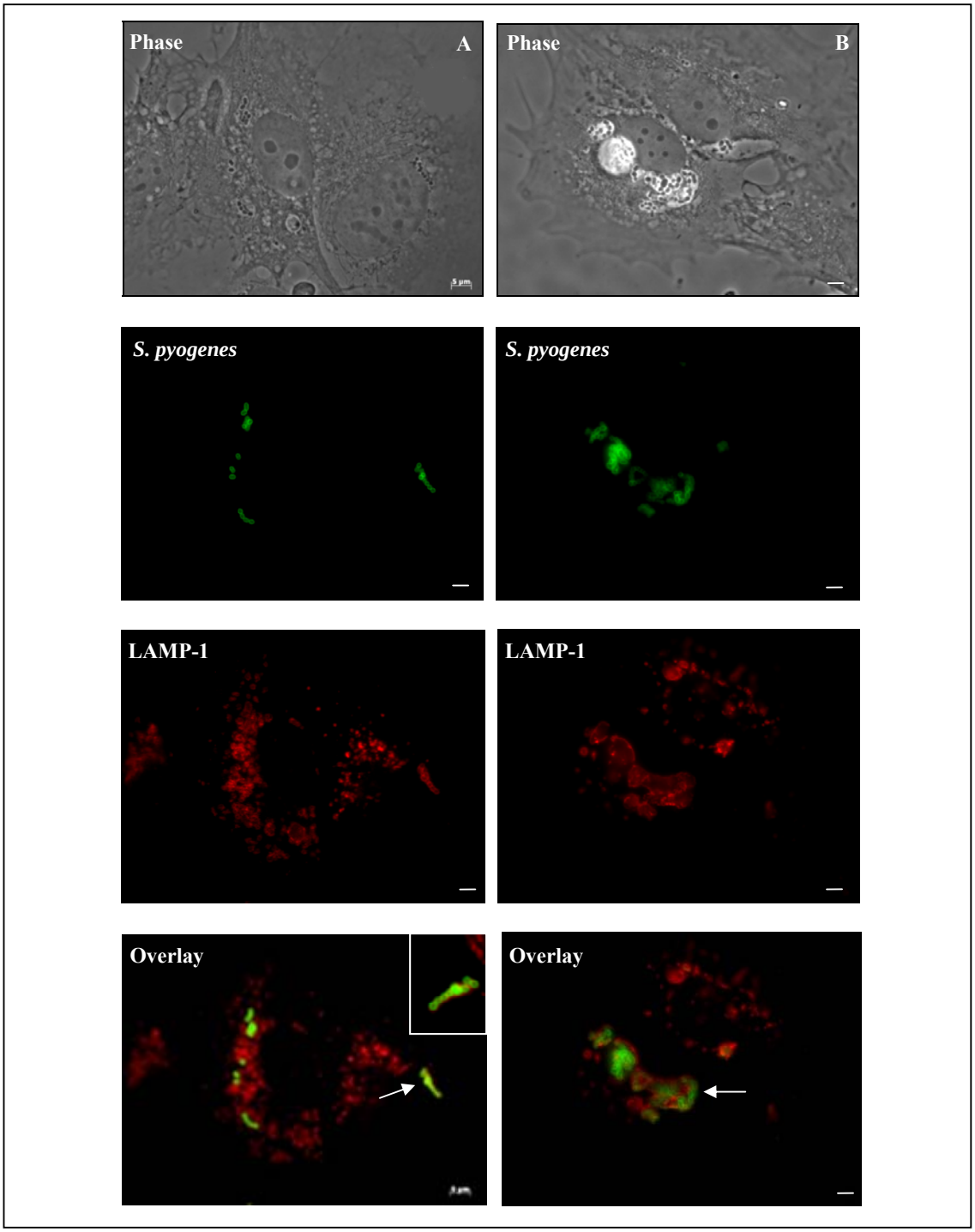


Figure 37. WT A475 and isogenic Δ*scpC* mutant reside in late endosomes/lysosomes. Cells were infected with WT A475 (A) and its isogenic Δ*scpC* mutant (B) for 2 h at 37°C with bacteria to cell ratio of 16:1. Infected cells were incubated with mouse monoclonal antibody against LAMP-1 for 1 h. To visualize the lysosomes, infected cells were then incubated with anti-mouse Alexa fluor® 568 (red) conjugated antibody. *S. pyogenes* were visualized by use of a polyclonal rabbit antiserum specific against *S. pyogenes* and with anti-rabbit Alexa fluor® 488 (green) conjugated secondary antibody. Enlarged area shows the co-localization of WT A475 with LAMP-1. Arrows delineate the co-localization of WT A475 and isogenic Δ*scpC* mutant with late endosomal/lysosomal marker LAMP-1. Each bar represents 5 µm.

4 Discussion

S. pyogenes is a highly specific human pathogen which can cause diseases ranging from minor infections to life threatening invasive deep tissue infections. *S. pyogenes* expresses several virulence proteins which participate in evading the host innate immune response towards infection via impairing bacterial clearance by phagocytes. The expression of virulence determinants is highly regulated, in order to express certain virulence proteins when required, according to the conditions within the host. Various studies have reported the correlation between the pathophysiology of invasive deep tissue infections and a marked reduction in the infiltration of neutrophils at the site of infection (Bakleh et al., 2005; Chong and Burrows, 2002; Cockerill et al., 1998b; Taylor et al., 1999). Recent findings have shown that *S. pyogenes* expresses a large protein, ScpC, which is responsible for the lack of acute inflammatory response in the local area of infection due to its ability to cleave CXC chemokines (IL-8, MIP-2, GCP-2 and GROα) (Hidalgo-Grass et al., 2006; Sumby et al., 2008). ScpC specifically cleaves IL-8, a major chemoattractant of neutrophils, between Gln^{59}-Arg^{60} in its C-terminal α-helix. This cleavage renders IL-8 biologically inactive, preventing neutrophil activation and infiltration into the infected tissue (Edwards et al., 2005; Zinkernagel et al., 2008). Experimental evidences have pointed towards the potential role of ScpC in the lack of acute inflammatory response frequently observed in necrotizing fasciitis patients. ScpC-mediated chemokine degradation have been implicated in the paucity of neutrophil recruitment in the mouse model of soft tissue streptococcal infection (Hidalgo-Grass et al., 2006). In addition to that, the marked upregulation in the expression of ScpC by invasive isolate M1T1 have further validated the significant contribution of ScpC in *S. pyogenes* pathogenesis (Sumby et al., 2006). These findings instigated us to further evaluate the role of this virulence protein in *S. pyogenes* pathogenesis of invasive infections.

It has been well recognized that *S. pyogenes* are capable of invading and surviving within cultured human epithelial and endothelial cells (Greco et al., 1995; Rohde et al., 2003; Talay et al., 2000). Invasion and transcytosis across host cells might represent the mechanism whereby *S. pyogenes* is able to enter the bloodstream and disseminate to the other tissues**.** Previous studies have reported that *S. pyogenes* are able to invade endothelial cells (Greco et al., 1995; Talay et al., unpublished; A. Nerlich, PhD thesis, 2006), however, the factors responsible for mediating the adherence to and invasion of endothelial cells by *S. pyogenes* remains unidentified. Studies showing the potential role of ScpC in retarding the IL-8-

mediated transendothelial migration and recruitment of neutrophils into the infected tissue (Edwards et al., 2005; Zinkernagel et al., 2008) prompted us to investigate the interaction of ScpC with human endothelial cells. In the present study, we demonstrate that ScpC plays an essential role in the invasion process of M3 serotype *S. pyogenes* into human umbilical vein endothelial cells, in addition to cleaving the host-derived chemokines. We also show that, despite the observation that ScpC is secreted in the culture supernatant, it is also a cell surface located protein, which is able reassociate with the bacterial surface after being secreted into the extracellular environment. Additionally, this study was designed to identify the essential domains required for the functional activity of ScpC. Previous studies have shown the IL-8 degrading activity of partially purified ScpC (also known as SpyCEP), which was isolated from the culture supernatant of *S. pyogenes* (Edwards et al., 2005). Although, it was highly desirable to have pure, biologically active ScpC to study the role of ScpC in *S. pyogenes* virulence, the active recombinant ScpC have been difficult to produce due to the large size and muti-domain structure of ScpC. In the present study, we have expressed the active recombinant full-length ScpC to aid the characterization of this important virulence factor.

4.1 Localization of ScpC on the *S. pyogenes* cell surface

The essential role of bacterial surface-anchored proteins in pathogenesis have been described by various reports (Lindahl et al., 2005; Niemann et al., 2004). In *S. pyogenes*, several cell wall-anchored proteins including the well known virulence factors M protein and Sfb1 have been shown to interact with host factors. Most of the surface-anchored proteins of *S. pyogenes* are multifunctional which can mediate the adherence to and invasion of host cells, binding to host cell extracellular proteins and/or resistance to phagocytosis by diverse strategies (Cunningham, 2000). Earlier studies have detected ScpC protease in the culture supernatant of *S. pyogenes* indicating that despite the presence of a C-terminal hydrophobic domain containing a cell wall-anchoring consensus motif (LPXTG) of Gram-positive bacteria, ScpC is secreted by *S. pyogenes* (Hidalgo-Grass et al., 2006; Edwards et al., 2005; Sumby et al., 2008). However, the localization of ScpC on the *S. pyogenes* cell surface and the significance of cell surface-anchoring have not been previously described. By using highly reactive ScpC specific antiserum, it could be demonstrated in this study that ScpC is located on the cell surface of *S. pyogenes*. The cell surface localization of ScpC was revealed by both immunogold electron microscopy and fluorescence microscopy. The recognition of cell surface localized ScpC by anti-ScpC antibodies suggests that ScpC is exposed on the surface

of *S. pyogenes* and thus may allow the protein to interact directly with host cell receptors or extracellular matrix proteins to mediate the interaction of *S. pyogenes* with the host cells**.** Since the culture supernatant of *S. pyogenes* was able to cleave CXC chemokine, it can be anticipated that the degradation of host-derived chemokines does not require bacterial interaction with chemokines. However, the surface exposed ScpC may facilitate the direct interaction of ScpC with CXC chemokines to inactivate the chemokines in near vicinity of the bacterium.

4.2 Reassociation of ScpC with the *S. pyogenes* cell surface

S. pyogenes express many anchorless glycolytic proteins such as surface dehydrogenase (SDH) and surface enolase (SEN), which were shown to be localized on the cell surface via reassociation. These surface-associated proteins bind to the serum protein plasminogen and retain the protease activity on the surface of *S. pyogenes* which may potentially contribute to tissue invasion and the overall pathogenicity of *S. pyogenes* (Pancholi and Fischetti, 1997a). Bergmann et al. (2001) have shown that the secreted enloase (Eno) protein, a plasminogen binding protein of *Streptococcus pneumoniae* is displayed on the cell surface via reassociation. Similar to *S. pyogenes*, the binding of plasminogen on the cell surface of *S. pneumoniae* via Eno provides the proteolytic activity on the cell surface which may represent the mechanism whereby *S. pneumoniae* facilitate the penetration of bacteria through the extracellular matrix thus enhancing tissue invasion during invasive pneumococcal infections (Bergmann et al., 2003; Kolberg et al., 2006). SDH acts as a virulence determinant of *S. pyogenes* and plays an important role in the invasion of *S. pyogenes* into host cells. Cell wall-bound SDH facilitates the interaction with the host cell receptors or other extracellular matrix components to trigger a signal transduction pathway via tyrosine phosphorylation of pharyngeal cell proteins, leading to the internalization of bacteria by host cells (Pancholi and Fischetti, 1997b). In this study, we demonstrated that ScpC can reassociate to the cell surface of *S. pyogenes* after being secreted into the extracellular environment as shown by the association of ScpC with the cell surface of both JS95 Δ*scpC* and A475 Δ*scpC* mutant strains after co-incubation with the culture supernatant of ScpC expressing wildtype (WT) JS95**.** Reassociation of ScpC onto the cell surface of JS95 Δ*scpC* and A475 Δ*scpC* mutant was confirmed by immunoelectron microscopy. Although the mechanism of reassociation is undetermined, the present study indicate that reassociation of ScpC with *S. pyogenes* cell

surface is not due to homotypic interaction, since binding back of ScpC has been demonstrated by using Δ*scpC* mutant strains.

This interesting observation shows correlation with the previous findings of Zinkernagel et al. (2008) who have shown that the presence of ScpC expressing *S. pyogenes* strain is able to protect other vulnerable bacteria in the local environment. This has been shown by isolation of similar number of surviving M1T1 Δ*scpC* and WT M1T1 from the lesion generated after co-infecting the mice with the equal amount of these strains at the same lesion site. Since M1T1 Δ*scpC* mutant was observed to be less virulent and susceptible to killing when injected alone, these findings suggested that WT M1T1 *S. pyogenes* might have promoted the survival of M1T1 Δ*scpC* mutant when co-infected in the same lesion. Also, the presence of ScpC expressing M1T1 *S. pyogenes* facilitated the survival of vancomycin resistant *Enterococcus faecalis* in a murine model of polymicrobial infection. Although, the mechanism behind this protective ability of ScpC expressing *S. pyogenes* is undefined, the current concept suggested the chemokine cleavage by ScpC and subsequent destruction of chemokine gradient as a principle mechanism. However, the present data provide a new insight into this phenomenon, suggesting that the enhanced survival of M1T1 Δ*scpC* mutant and other species of bacteria in the presence of ScpC expressing strain may be attributed to the reassociation of the extracellular secreted ScpC on the surface of bacteria, which might protect bacteria either via mediating the enhanced chemokine degradation or by enabling the bacteria to interact with putative host cell receptors to colonize and invade the host cells. Thus, the present data indicate a distinct underlying mechanism for the *trans* action of ScpC to facilitate the survival of other bacteria in the local environment of infection.

4.3 Potential role of ScpC in the invasion process of M3 *S. pyogenes*

Further, we investigated the hypothesis that surface located ScpC might facilitate the interaction of *S. pyogenes* with host endothelial cells to mediate adherence to and invasion into endothelial cells. Invasion of *S. pyogenes* into host cells may contribute to the enhanced pathogenicity of *S. pyogenes* by providing an intracellular niche to protect bacteria from immune defense mechanisms and antibiotic therapy. Various studies have suggested that the ability of *S. pyogenes* to invade the host cells might contribute to the frequent failure of antibiotics to eradicate infection (Osterlund and Engstrand, 1997; Sela et al., 2000). In addition, the invasion and transmigration of epithelial and endothelial layers seem to be a precondition for the invasiveness of *S. pyogenes*. *S. pyogenes* expresses multiple cell surface-

anchored adhesins and invasins, which mediate adherence to and invasion of bacteria into host cells (Kreikemeyer et al., 2004). The initial step of establishing infection is attachment to and colonization of host cells by *S. pyogenes* which is mediated by adhesins. Adhesive proteins interact with surface receptors of host cells, which are often involved in cell-matrix or cell-cell adherence. Many adhesins also act as invasin triggering the uptake of bacteria into host cells. The interaction of an adhesin with its host cell receptor triggers a cascade of signals within the host cells, subsequently leading to the internalization of bacteria into host cells. (Courtney et al., 2002). Many adhesins and invasins of *S. pyogenes* involved in the attachment and invasion of epithelial cells have been identified including Sfb1, M protein and C5a peptidase. However, the data concerning the virulence determinants involved in the invasion of *S. pyogenes* into endothelial cells is very limited.

Endothelial cells line the interior surface of arteries, veins and post-capillary venules in the skin, forming an interface between blood and the underlying tissue. It has been widely reported that endothelial derived cytokines contribute significantly to the occurrence of inflammatory and immunological responses towards infection (Krishnaswamy et al., 1999). Breaching an endothelial barrier and/or modulation of endothelial function by *S. pyogenes* might contribute significantly to the outcome of invasive streptococcal infection. Many bacterial pathogenes such as *Streptococcus agalactiae* (GBS), *Staphylococcus aureus* and *Neisseria meningitidis* are able to invade endothelial cells (Menzies and Kourteva, 1998; Nassif et al., 2002). Reports showing the invasion into and transmigration of group B *Streptococcus* (GBS) across a cultured monolayer of immortalized human brain microvascular endothelial cells (BMEC) suggest that the invasion of GBS into the endothelial monolayer comprising the blood brain barrier can significantly contribute to the pathogenesis of GBS meningitis in neonates (Gibson et al., 1993; Nizet et al., 1997). Previous studies have shown that M3 *S. pyogenes* are able to invade cultured human endothelial cells, via an internalization process involving the generation of host cell membrane protrusions around the streptococci to engulf the bacteria into the cells (Greco et al., 1995; Nerlich et al., unpublished).

The present study indicates that ScpC plays an essential role in the invasion of M3 serotype *S. pyogenes* strain A475, isolated from a patient with invasive disease, into the cultured human umbilical vein endothelial cells (HUVEC). The *scpC* deletion mutant of M3 *S. pyogenes* strain A475 showed significantly reduced ability to invade endothelial cells in comparison to the WT A475, at the initial time points of infection (30 min post-infection). The growth rate of WT A475 and its isogenic Δ*scpC* mutant was similar, explaining that the difference in the

invasion of A475 and its isogenic Δ*scpC* mutant is not a result of a difference in the growth behavior of the bacteria. ScpC *per se* was able to trigger the invasion process. This was indicated by the efficient adherence to and subsequent internalization of purified recombinant ScpC coated latex beads by HUVEC. Late endosomes/lysosomes were identified as terminal intracellular compartment of ScpC coated beads as indicated by the co-localization of ScpC coated beads with the late endosomal/lysosomal marker LAMP-1 at 2 h post-incubation. Efficient internalization of EC-PR (putative catalytic domain containing catalytic triad Asp-His-Ser) coated beads by HUVEC indicated that the minimal domain of ScpC required to mediate the invasion of endothelial cells is restricted to the N-terminal PR domain.

However, since the uptake of A475 Δ*scpC* mutant by HUVEC was not completely abolished, it cannot be excluded that other factors are also involved in the invasion of *S. pyogenes* into endothelial cells. Similar to the invasion mechanism of other bacteria such a *Yersinia*, *Listeria* and *Shigella* ssp. (Cossart et al., 2003; Cossart and Sansonetti, 2004), the *S. pyogenes* adhesion to and invasion into the host cells can be mediated by multiple cell surface-bound factors (Courtney et al., 2002). It might be possible that ScpC, along with other invasins, mediates the invasion of *S. pyogenes* into endothelial cells. Interestingly, the results of this study using M3 *S. pyogenes* and endothelial cells differ from the previous report showing the enhanced adherence to and invasion of M1T1 Δ*scpC* mutant strain into human pharyngeal epithelial cells (HEp-2) in comparison to WT M1T1 *S. pyogenes* (Zinkernagel et al., 2008). It is likely that epithelial cells do not express the receptors which are required for the interaction with ScpC. This may explain the specific interaction of ScpC with endothelial cells but not with epithelial cells.

Study of the kinetics of invasion of WT A475 and its isogenic Δ*scpC* mutant strain into HUVEC showed that the WT A475 established contact and started invading endothelial cells within 15 min of infection and many intracellular bacteria were observed at 30 min post-infection. However, as incubation time was increased (2 h post-infection) the number of intracellular WT A475 decreased, while the number of intracellular A475 Δ*scpC* mutant increased drastically in a subpopulation of cells. It has been previously shown that M3 *S. pyogenes* can invade and subsequently promote its transcytosis across human endothelial cells via exocytosis of lysosomes (Talay et al., unpublished). Although lysosomes are considered as terminal compartments of the endocytic pathway, studies have shown that lysosomes can act as exocytotic vesicles (Gerasimenko et al., 2001). Regulated lysosomal exocytosis is a general physiological mechanism in all the cells in response to the membrane injury in order to reseal the membrane of injured cells (Reddy et al., 2001; Roy et al., 2004). Hakansson et al. (2005)

have also shown that damage to the keratinocyte plasma membrane by *S. pyogenes* virulence protein, streptolysin O (SLO) triggers the mobilization of lysosomes to the cell surface to fuse with the damaged areas of the membrane as a repair mechanism. In agreement with the previous studies of Talay et al. (unpublished), the present data indicated that after 1 h infection of HUVEC with WT A475, phagosomes containing A475 fused with LAMP-1 positive compartments. At 2 h post-infection, a very few intracellular WT A475 were observed, although the WT A475 that were found intracellularly were observed to co-localize with LAMP-1 on the cell surface indicating that A475 escape from the endothelial cells via lysosomal exocytosis (Talay et al., unpublished; N. Petermann, Diploma thesis, 2003)**.** However, A475 Δ*scpC* mutant confined inside the LAMP-1 positive compartments at 2 h post-infection. Based on these observations, our data suggests that the A475 Δ*scpC* mutant accumulated inside the cells possibly due to imparied exocytosis and thus mutant strain are unable to escape from endothelial cells. The role of ScpC in the transcytosis of *S. pyogenes* through endothelial monolayer can be further studied by analysing if the ability to transmigrate the endothelial cells is imparied in A475 Δ*scpC* mutant, using a transwell system.

The accumulation of A475 Δ*scpC* in a subpopulation of endothelial cells may be attributed to the fact that the receptor involved in the internalization of *S. pyogenes* was expressed by a subset of cells. Heterogeneity among the host cells due to non-homogenous expression of the specific receptors involved in the interaction with other *S. pyogenes* invasins may explain why only subpopulation of cells were invaded by *S. pyogenes*. Damage to the host cell membrane by *S. pyogenes* virulence proteins can trigger lysosomal exocytosis thus releasing the lysosomal content to the extracellular environment (Hakansson et al., 2005), which might result in an acidic microenvironment in the host during streptococcal infection. This hypothesis correlates with the observation that an acidic environment triggered the interaction of ScpC with host cells. Also, it is likely that ScpC-mediated interaction of *S. pyogenes* with endothelial cell might also contribute to enhance the invasiness of *S. pyogenes* via modulating the endothelial cell function. Endothelial cells produce many cytokines including IL-8 and IL-6 which are important in regulating the transmigration of leukocytes and the development of an inflammatory response (Chen and Manning, 1996). Since it has been previously described that the IL-8 expression by cultured endothelial cells was induced in response to the bacterial infections and endothelial derived IL-8 was sufficient to induce the *in vitro* transendothelial migration of neutrophils (Yao et al., 1996), it can be anticipated that endothelial-derived IL-8

may significantly contribute to facilitate the adhesion and transendothelial migration of neutrophils to sites of *S. pyogenes* infection.

In contrast to the results of infection assay, the antibiotic protection assay showed significant reduction in the number of intracellular A475 Δ*scpC* mutant at 2 h post-infection in comparison to the number of intracellular WT A475, as quantified by counting the viable colony forming units of these strains released by infected cells. The observed difference in the invasion of WT A475 and A475 Δ*scpC* mutant into HUVEC may be attributed to the difference in the intracellular survival of these strains. Several factors may have contributed to this difference. Firstly, enhanced membrane damage due to the accumulation of A475 Δ*scpC* mutant inside the cells might have caused the leakage of antibiotics into the cells, which ultimately resulted in reduced viability of the A475 Δ*scpC*. Secondly, there may have been the enhanced intracellular killing of A475 Δ*scpC* mutant by the bactericidal activity of lysosomes, in comparison to WT parental strain, which is able to escape from the endothelial cells. Alternatively, the intracellular A475 Δ*scpC* might have induced the cell death releasing the intracellular bacteria free in the culture medium and exposed to the antibiotics in the medium. Marouni and Sela (2004) have shown that the internalization of *S. pyogenes* into epithelial cells induced the apoptosis of host cells suggesting that the induction of host cell death may be a means of releasing essential nutrients required for the bacterial growth. Many bacterial pathogenes such as *Staphylococcus aureus* and *Shigella flexneri* are capable of inducing the host cell death via apoptosis (Weinrauch and Zychlinsky, 1999). The intracellular *S. aureus* have also been shown to damage the endothelial cell monolayer by affecting the membrane integrity and consequently induced the apoptosis of endothelial cells (Menzies and Kourteva, 1998).

Furthermore, this study demonstrated that the adherence to and internalization of ScpC coated latex beads by HUVEC was blocked by pre-incubation of ScpC coated beads with purified anti-ScpC IgG, indicating that the interaction of ScpC with endothelial cells can be efficiently blocked with anti-ScpC antibody, thus suggesting that specific antibody induced against ScpC can be a tool to inhibit the invasion mediating activity of ScpC. This finding additionally indicates the specificity of the interaction of ScpC with endothelial cells and suggests that ScpC interacts specifically with a receptor on HUVEC which is potentially involved in the entry of *S. pyogenes* into endothelial cells. Whereas, the pre-incubation of ScpC with anti-ScpC antibody did not considerably effect its IL-8 proteolytic activity, indicating that different epitopes are involved in the IL-8 proteolytic activity and the invasion mediating activity of ScpC. Lei et al. (2000) have reported that patients with pharyngitis and invasive

diseases had specific antibodies against ScpC, indicating that ScpC is highly immunogenic in humans. Furthermore, a report showing that specific antibodies to ScpC are protective against streptococcal infection in mice (Rodriguez-Ortega et al., 2006) supports the biological significance of the surface location of ScpC and suggests that specific antibodies against ScpC are protective either due to its ability to opsonize *S. pyogenes* or by interfering with ScpC function. Inhibiting the invasion of *S. pyogenes* into host cells may as well play a significant role in blocking the invasiveness of *S. pyogenes*, thus anti-ScpC antibodies may prove to have a therapeutic use. However, preliminary results of antibiotic protection assay indicate that pre-incubation of *S. pyogenes* A475 with purified anti-ScpC antibodies did not substantially reduce *S. pyogenes* adherence to and invasion into HUVEC (data not shown).

S. pyogenes have evolved multiple mechanisms to invade a wide variety of host cells. Sfb1-mediated adherence to and invasion of *S. pyogenes* into epithelial cells involves specific interaction between the C-terminal fibronectin-binding repeat region of cell surface-bound Sfb1 protein and the extracellular matrix protein fibronectin, which acts as a bridge between bacteria and integrin receptor $\alpha_5\beta_1$ present on host epithelial cells (Molinari et al., 1997). Rohde et al. (2003) have shown that Sfb1-expressing *S. pyogenes* follow a caveolae-mediated endocytic pathway to gain entry into epithelial as well as endothelial cells via fibronectin-mediated interaction of Sfb1 with the cellular integrin $\alpha_5\beta_1$. This interaction triggers the formation of membrane invaginations in the host cells, which leads to the internalization of the bacteria. In addition, the major virulence determinant of *S. pyogenes*, M1 protein mediates invasion of *S. pyogenes* into epithelial cells via binding to fibronectin which in turn binds to the integrins on host cells. A nonpeptide antagonist of integrin $\alpha_5\beta_1$ inhibits fibronectin-mediated invasion of *S. pyogenes* into A549 as well as human tonsillar epithelial cells. However, it does not block the invasion of *S. pyogenes* via laminin-mediated interaction with $\alpha_3\beta_1$ in lung epithelial A549 cells, suggesting that the interaction of bacterial cell surface-bound M1 protein with multiple integrin sites may lead to the invasion of *S. pyogenes* into host cells (Cue et al., 1998; Cue et al., 2000).

In order to identify the mechanism of ScpC-mediated invasion of *S. pyogenes* into endothelial cells, we tested the binding of the extracellular matrix proteins, fibrinogen, plasminogen and collagen with recombinant ScpC. *In vitro* analysis using recombinant ScpC indicated that ScpC did not bind to any of these extracellular matrix proteins on our experimental setup. Furthermore, a monoclonal antibody directed against human integrin β_1 did not affect the adherence to and internalization of ScpC coated beads by endothelial cells. Intergrin β_1 is a subunit of the heterodimeric eukaryotic cell surface receptor for various extracellular matrix

proteins including laminin, fibrinogen and collagen. This indicates that ScpC-mediated invasion of *S. pyogenes* into endothelial cells is independent of the potential to interact with these extracellular matrix proteins or with β_1 integrin**.** Therefore, the identification of the receptor which is involved in the interaction of ScpC with endothelial cells requires further analysis.

By recombinantly expressing several distinct domains of ScpC in *E. coli*, the functional domains of ScpC required for the IL-8 proteolytic activity were assessed. For characterization of the IL-8 degrading domain of ScpC, we investigated whether the putative catalytic domain (PR-domain) of ScpC, identified based on the homology to existing serine proteases, is essential and sufficient for its proteolytic activity. By monitoring the IL-8 degradation by recombinant fusion protein constructs encompassing full-length ScpC and distinct domains of ScpC, either expressed independently or with flanking domains, the results indicated that the recombinant full-length ScpC was functionally active to cleave IL-8 *in vitro*. The smaller fragment of ScpC, EC-PR+A encompassing the PR domain and the A-domain was sufficient to cleave IL-8. However, the PR-domain alone did not degrade IL-8, indicating that the PR-domain is proteolytically inactive. Previous studies of Siezen et al. (1999) have shown that each domain of extracellular cell envelope proteases has a distinct function. The A-domain is conserved among most of extracellular cell envelope protease of lactic acid bacteria and it has been shown to play a role in defining the substrate specificity of PrtP, a cell envelope serine proteinase of *Lactococcus lactis* (Siezen et al., 1993). In line with this observation in *L. lactis*, the present data suggests that the A-domain is essential for the proteolytic activity of ScpC and acts in concert with the PR-domain for the cleavage of IL-8. Whereas, experiments performed to localize the minimal domain involved in the invasion mediating activity of ScpC showed that the PR-domain is sufficient for the adherence and internalization of ScpC coated latex beads by HUVEC suggesting that the invasion mediating activity of ScpC is independent of its proteolytic activity.

5 Summary and Outlook

In summary, ScpC is a multi-functional cell surface located protein of *S. pyogenes* which significantly contributes to the invasion and transcytosis of M3 *S. pyogenes* through the endothelial monolayer. In addition to the contribution of the chemokine degrading proteolytic activity of ScpC, the invasion associated activity of ScpC might also have a substantial impact on *S. pyogenes* pathogenesis. This study provides valuable information about the properties of ScpC by defining the domains required for its functional activity, which may prove useful in understanding the complex pathogenic properties displayed by this virulence protein of *S. pyogenes.* Specific antibodies raised against ScpC demonstrated the inhibitory effect towards the adherence and internalization of ScpC coated beads by HUVEC, suggesting that the specific antibodies against ScpC may provide a useful tool in the prevention of the invasion of *S. pyogenes* into endothelial cells. Additionally, this study demonstrated that ScpC is able to reassociate to the *S. pyogenes* cell surface after being secreted into the extracellular environment, which may have a significance in facilitating the survival and dissemination of the bacteria in the host. Based on the multi-functional role of ScpC in *S. pyogenes* virulence and conserved nature of *scpC* in distinct M serotypes of *S. pyogenes*, it can be a potential target for use as a novel therapeutic agent against invasive *S. pyogenes* infections. Further investigations will involve the identification of the receptors required in the ScpC-mediated interaction of *S. pyogenes* with endothelial cells which can help in understanding the invasion mechanism of M3 *S. pyogenes*.

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7 Appendix

7.1 List of Abbreviations

A	Absorbance
Amp	Ampicillin
APS	Ammonium persulfate
BSA	Bovine serum albumin
cfu	Colony-forming units
dH_2O	Distilled water
DMEM	Dulbecco's Modified Essential Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonulcease
dNTP	Deoxyribonulceotide-5'-phosphate
EDTA	Ethylenediamine tetraacetic acid
EGM	Endothelial cell growth medium
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Fn	Fibronectin
GCP-2	Ganulocyte chemotactic protein 2
GROα	Growth related oncogene α
GST	Glutathione S-transferase
h	Hour
His	Histidine
HPLC	High performanceliquid chromatography
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IgG	Immunoglobulin G
IL-8	Interleukin 8
IPTG	Isopropyl-b-D-thiogalactopyranoside
Kan	Kanamycin
LB	Luria-Bertani
MALDI	Matrix assisted laser desorption/ionization

min	Minute
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate buffer saline with 0.05% tween
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethansulfonylfluorid
rScpC	Recombinant ScpC
RT	Room temperature
ScpA	C5a peptidase
ScpC	Streptococcal chemokine protease C
SD	Standard deviation
SDS	Sodiumdodecylsulfate
sec	Second
Sfb	Streptococcal fibrinectin binding protein
SLO	Streptolysin O
SLS	Streptolysin S
TCS	Two-component system
TEMED	Tetramethylethylenediamine
THY	Todd-hewitt broth with yeast extract
Tris	Trishydroxymethylaminomethane
TSB	Tryptic soy broth
U	Units
UV	Ultraviolet light
v/v	Volume per volume (volume percentage)
w/v	Weight per volume (weight percentage)
WT	Wildtype

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